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Jc530 U.S. PTO

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December 4, 1997

Attorney Docket No.: 06868/005002

## Box Patent Application

Assistant Commissioner of Patents  
Washington, DC 20231

Presented for filing is a new continuation patent application of:

BOSTON  
HOUSTON  
NEW YORK  
SOUTHERN CALIFORNIA  
SILICON VALLEY  
TWIN CITIES  
WASHINGTON, DC

Applicant: ANTHONY J.F. D'APICE, MARTIN J. PEARSE, ALLAN J. ROBINS, ROBERT J. CRAWFORD  
Title: MATERIALS AND METHODS FOR MANAGEMENT OF HYPERACUTE REJECTION IN HUMAN XENOTRANSPLANTATION

Enclosed are the following papers, including those required to receive a filing date under 37 CFR §1.53(b):

	<u>Pages</u>
Specification	133
Claims	8
Abstract	1
Declaration	4
Drawing(s)	39

### Enclosures:

- Rule 63 declaration, copy from a previous application under rule 63(d) for continuation or divisional only.
- Preliminary amendment, 1 page.
- Postcard.

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Date of Deposit December 4, 1997  
I hereby certify under 37 CFR 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office To Addressee" with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Donna Q. Taylor  
Donna Q. Taylor

FISH & RICHARDSON P.C., P.A.

December 4, 1997

Page 2

This application is a continuation (and claims the benefit of priority under 35 USC 120) of U.S. application serial no. 08/378,617, filed 01/26/95. The disclosure of the prior application is considered part of (and is incorporated by reference in) the disclosure of this application.

Preliminarily, on page 1 of the specification, before line 1, insert --This is a continuation of U.S. application serial no. 08/378,617, filed 01/26/95, (pending).--

Under 35 USC §120, this application claims the benefit of prior U.S. application 08/378,617, filed 01/26/95.

Please delete Peter D. Rathjen as a named inventor in this new continuation patent application pursuant to §1.53(d)(4).

Basic filing fee	790.00
Total claims in excess of 20 times \$22.00	22.00
Independent claims in excess of 3 times \$82.00	164.00
Fee for multiple dependent claims	0.00
Total filing fee:	\$ 976.00

A check for the filing fee is enclosed. Please apply any other required fees or any credits to deposit account 06-1050, referencing the attorney docket number shown above.

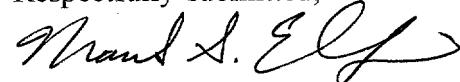
If this application is found to be incomplete, or if a telephone conference would otherwise be helpful, please call the undersigned at 612/335-5070.

Kindly acknowledge receipt of this application by returning the enclosed postcard.

Please send all correspondence to:

Mark S. Ellinger  
60 South Sixth Street, Suite 3300  
Minneapolis, MN 55402

Respectfully submitted,



Mark S. Ellinger, Ph.D.  
Reg. No. 34,812  
Enclosures

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : D'Apice et al. Art Unit: Unknown  
Serial No.: Unknown Examiner: Unknown  
Filed : December 4, 1997  
Title : MATERIALS AND METHODS FOR MANAGEMENT OF HYPERACUTE  
REJECTION IN HUMAN XENOTRANSPLANTATION

Assistant Commissioner for Patents  
Washington, DC 20231

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows:

In the Claims

Please cancel claims 8-11, 13-14, 19-20, 27, 30, 32-45.

REMARKS

Claims 8-11, 13-14, 19-20, 27, 30, 32-45 have been canceled. Claims 1-7, 12, 15-18, 21-26, 28-29 and 31 remain for consideration.

Please apply any charges not covered, or any credits, to Deposit Account 06-1050.

Respectfully submitted,

Date: December 4, 1997



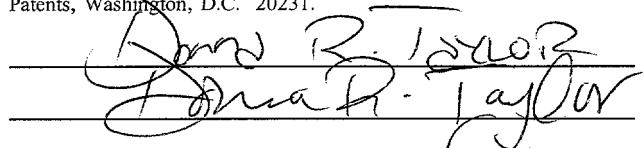
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APPLICATION  
FOR  
UNITED STATES LETTERS PATENT

**TITLE:** MATERIALS AND METHODS FOR MANAGEMENT OF  
HYPERACUTE REJECTION IN HUMAN  
XENOTRANSPLANTATION

**APPLICANT:** ANTHONY J.F. D'APICE, MARTIN J. PEARSE, ALLAN J.  
ROBINS, ROBERT J. CRAWFORD AND PETER D. RATHJEN

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Kathleen Bowen

Kathleen Bowen

**MATERIALS AND METHODS FOR MANAGEMENT OF HYPERACUTE  
REJECTION IN HUMAN XENOTRANSPLANTATION**

**Cross Reference to Related Applications**

5 The present application is a continuation-in-part of U.S. Application Serial No. 08/188,607, filed January 27, 1994.

**Field of the Invention**

This invention relates generally to the field of 10 xenotransplantation. In particular this invention relates to methods and materials for reduction or elimination of the hyperacute rejection response in humans. More particularly, this invention relates to methods for treating human serum to reduce or eliminate 15 hyperacute rejection. This invention also relates to methods and materials for generating non-human organs lacking or having reduced  $\alpha$  1,3 galactosyl transferase activity.

**Background of the Invention**

20 It is widely acknowledged that there is an acute, worldwide shortage of human organs for transplantation. This is in spite of legislative changes and education programs to increase public awareness of the problem. In the United States, for example, there is an estimated 25 annual shortfall of approximately 18,000 kidneys/year. Similarly, in Australia in 1992, only 41% of renal patients awaiting transplantation received transplants.

In Japan the imbalance between supply and demand is even greater due to religious prohibitions on the use of organs from cadaveric donors.

The benefits of transplantation can be seen by 5 comparing the rehabilitation rates of transplant patients with those of dialysis patients. In Australia and New Zealand, the majority of transplant patients (60%) are capable of full time work or school with a further 10% in part time work, while only 7% are unfit for work. In 10 contrast, 23% of dialysis patients are capable of full time work or school, with 15% involved in part time work and 20% unfit for work. The remainder are "retired." Fifteenth Report of the Australia and New Zealand Dialysis and Transplant Registry (ANZDATA), Queen 15 Elizabeth Hospital, Woodville, S.A., APS Disney, ed. (1992).

The direct financial cost of dialysis in Australia and New Zealand is approximately \$A45,000/patient/year. In addition, indirect costs due to unemployment and 20 sickness are higher in dialysis patients and the social costs are considerable. Transplantation engenders an expense of approximately \$A30,000/patient in the first year and \$A14,000/patient/year thereafter. These statistics indicate that a) transplantation is the 25 optimal therapy for end stage renal failure; b) there is an undersupply of donor kidneys; and c) present strategies aimed at increasing the transplant rate have

been less than successful. There are, in addition, serious shortages of other transplantable organs including hearts, livers, lungs and pancreases.

The use of xenografts (transplants between 5 species) is one option for overcoming the short supply of human organs for transplantation. Non-viable, non-antigenic xenografts are commonly used in vascular reconstruction (bovine arteries) and in cardiac surgery (porcine cardiac valves). However, despite their 10 occasional use in the past, immunological barriers have prevented the common use of viable xenografts. Between 1964 and 1991 a total of 27 non-human primate to human organ xenografts was reported; the longest reported patient survival was 9 months. Two liver transplants 15 from baboon to human were recently performed in anticipation that modern immunosuppressive therapies could cope with the severe rejection problems likely to occur in xenotransplantation. To date, the course of one of these patients has been reported, and in this case 20 rejection was not the direct cause of death. Starzl et al., Baboon-to-Human Liver Transplantation. Lancet 341: 65-71 (1993). This clinical experience indicates that a) non-human organs can function and support human life; b) rejection episodes can be reversed by conventional anti- 25 rejection therapy; and c) the mechanisms of rejection are similar, in principle, to those in allograft rejection.

It is unlikely that primates will be a satisfactory source of organs for xenotransplantation. Most are endangered species, breed slowly in the wild and poorly in captivity. The baboon is an exception to these 5 generalizations, but other disadvantages limit the usefulness of this species. Baboons have single pregnancies, long gestation times, are difficult and expensive to maintain and may be infected with or carry organisms, particularly viruses, that are pathogenic in 10 humans. For hearts and kidneys where organ size may be a consideration, the smaller primates are unsatisfactory as donors to human adults. Finally, the use of primates is likely to arouse considerable opposition from the public.

These difficulties have led to renewed interest in 15 the use of non-primate species as organ donors for human patients. The pig is a widely acknowledged choice for xenotransplantation into humans. The pig erythrocyte diameter ( $6.5\mu\text{m}$ ) and, by implication, its capillary size, are similar to humans, facilitating connection of 20 xenografts to the human circulatory system. The pig breeds well in captivity, has a short gestation time and produces large litters. In addition, pigs can be bred and maintained in low pathogen facilities, can be reared to any size and do not arouse the level of public 25 reaction associated with primates.

The immunological barriers to use of pig organs in human patients include a) an immediate severe

("hyperacute") rejection phenomenon that develops in minutes to hours after transplantation, and b) a proposed acute rejection that develops in days to weeks. Once the hyperacute rejection phenomenon has been overcome, it is 5 expected that normal acute rejection would ensue. This form of rejection is thought to be similar to that experienced with allografts (transplants between individuals of the same species) and should be amenable to normal immunosuppressive therapies.

10 Both preformed "natural antibodies" (xenoantibodies) and complement regulating factors in human serum are thought to be involved in the process of hyperacute rejection. Hyperacute rejection is thought to be initiated when xenoantibodies bind to epitopes on the 15 endothelium of a donor organ, activating the classical complement pathway.

Summary of the Invention

A purified and isolated nucleic acid molecule of the present invention comprises the porcine nucleic acid 20 sequence depicted in Figure 4 (SEQ ID NO: 7), which encodes a porcine polypeptide having  $\alpha$ -1,3 galactosyltransferase activity. Variations on this sequence that may be routinely generated by the skilled artisan include those sequences corresponding to Figure 4 25 but varying within the scope of the degeneracy of the genetic code. That is, the present invention includes

variants of the sequence set out in Figure 4, readily determined by the skilled artisan, that code for the same amino acid sequence encoded by the sequence set out in Figure 4. The present invention also includes a 5 purified and isolated nucleic acid molecule that encodes a porcine  $\alpha$ -1,3 galactosyltransferase and that hybridizes under standard high stringency conditions with a sequence complementary to the sequence set out in Figure 4, or with a sequence complementary to a variation of the 10 sequence set out in Figure 4 within the scope of the degeneracy of the genetic code. The complementary strands to the above-described nucleic acid sequences are readily determined by standard methods, and are also within the scope of the present invention.

15 Within the parameters set out in the preceding paragraph, the present invention includes variants of the porcine  $\alpha$ -1,3 galactosyltransferase coding sequence that preserve the functional characteristics of the native gene product. Such variants include, for example, minor 20 nucleotide variations in the 5' untranslated region or in various coding regions of the disclosed sequence. Minor amino acid variations deriving from changes in the coding regions, that leave a functional  $\alpha$ -1,3 galactosyltransferase catalytic site, membrane anchor 25 domain and stem region as described below, are within the scope of the present invention. Such routine variations in nucleic acid and amino acid sequences can be

identified by those having ordinary skill in the art based on the sequence and structural information provided herein.

As used herein, "high stringency conditions" are 5 those hybridization conditions generally understood by the skilled artisan to reflect standard conditions of high stringency as set out in widely recognized protocols for nucleic acid hybridization. See, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual (2nd 10 Edition), Cold Spring Harbor Laboratory Press (1989), pp. 1.101 - 1.104; 9.47 - 9.58 and 11.45 - 11.57. Generally, these conditions reflect at least one wash of the hybridization membrane in 0.05x to 0.5x SSC with 0.1% SDS at 65°C, or washing conditions of equivalent stringency.

15 The present invention also includes a host cell transformed with any of the above-described purified and isolated nucleic acid molecules, as well as a porcine  $\alpha$ -1,3 galactosyltransferase encoded by such transforming nucleic acid molecules and expressed from the host cell.

20 Methods for transforming appropriate host cells and for expressing polypeptides from such host cells are known in the art and are described, for example, in Sambrook et al., (1984), pp. 12.2-12.44; 16.3-17.44.

The invention further includes a DNA construct 25 useful for inactivating the porcine  $\alpha$ -1,3 galactosyltransferase gene by insertion of a desired DNA sequence into an insertion site of the gene. As used

herein, the term " $\alpha$ -1,3 galactosyltransferase gene" includes the exons encoding or potentially encoding  $\alpha$ -1,3 galactosyltransferase, introns contiguous with such exons, and regulatory elements associated with such exons 5 and introns. The DNA construct includes the desired DNA sequence flanked by first and second homology sequences. These first and second homology sequences are sufficiently homologous, respectively, to first and second genomic sequences flanking the insertion site to 10 allow for homologous recombination of the DNA construct with the porcine  $\alpha$ -1,3 galactosyltransferase gene when the DNA construct is introduced into a target cell containing the porcine  $\alpha$ -1,3 galactosyltransferase gene. Preferably the insertion site is within exon 4, exon 7, 15 exon 8 or exon 9 of the porcine  $\alpha$ -1,3 galactosyltransferase gene. The desired DNA sequence is preferably a selectable marker, including but not limited to the neo<sup>R</sup> gene, the hydromycin resistance (hyg<sup>R</sup>) gene and the thymidine kinase gene. The desired DNA sequence 20 may be bordered at both ends by FRT DNA elements, with stop codons for each of the three reading frames being inserted 3' to the desired DNA sequence. Presence of the FRT elements allows the selectable marker to be deleted from the targeted cell, and the stop codons ensure that 25 the  $\alpha$ -1,3 galactosyltransferase gene remains inactivated following deletion of the selectable marker.

The invention further includes a DNA construct useful for inactivating the murine  $\alpha$ -1,3 galactosyltransferase gene by insertion of a desired DNA sequence into an insertion site of the gene. The DNA 5 construct includes the desired DNA sequence flanked by first and second homology sequences. These first and second homology sequences are sufficiently homologous, respectively, to first and second genomic sequences flanking the insertion site to allow for homologous 10 recombination of the DNA construct with the murine  $\alpha$ -1,3 galactosyltransferase gene when the DNA construct is introduced into a cell containing the murine  $\alpha$ -1,3 galactosyltransferase gene. Preferably the insertion site is within exon 4, exon 7, exon 8 or exon 9 of the 15 murine  $\alpha$ -1,3 galactosyltransferase gene. The desired DNA sequence is preferably a selectable marker, including but not limited to the neo<sup>R</sup> gene, the hyg<sup>R</sup> gene and the thymidine kinase gene. The desired DNA sequence may be bordered at both ends by FRT DNA elements, with stop 20 codons for each of the three reading frames being inserted 3' to the desired DNA sequence. Presence of the FRT elements allows the selectable marker to be deleted from the targeted cell, and the stop codons ensure that the  $\alpha$ -1,3 galactosyltransferase gene remains inactivated 25 following deletion of the selectable marker.

The invention also includes methods for generating a mammalian totipotent cell having at least one

inactivated (non-functional)  $\alpha$ -1,3 galactosyltransferase alleles, where the totipotent cell is derived from a mammalian species in which alleles for the  $\alpha$ -1,3 galactosyltransferase gene normally are present and 5 functional. A "functional" allele is capable of being transcribed and translated to produce a polypeptide having an activity the same as or substantially similar to the native  $\alpha$ -1,3 galactosyltransferase. The methods include providing a plurality of cells characterized as 10 totipotent cells of the aforementioned mammalian species, introducing into the totipotent cells a nucleic acid construct effective for inactivating the  $\alpha$ -1,3 galactosyltransferase gene by insertion of a desired DNA sequence into an insertion site of the gene through 15 homologous recombination, and then identifying a totipotent cell having at least one inactivated  $\alpha$ -1,3 galactosyltransferase allele.

The totipotent cells can include, without limitation, embryonic stem (ES) cells, primordial germ 20 cells (PGC's) and eggs. The cells can be taken from a variety of mammalian species in which alleles for the  $\alpha$ -1,3 galactosyltransferase gene are present and functional, including without limitation murine and porcine species.

25 The invention further includes methods for generating a mammal lacking a functional  $\alpha$ -1,3 galactosyltransferase gene, where the mammal belongs to a

species having a functional  $\alpha$ -1,3 galactosyltransferase gene. The methods include providing a mammalian totipotent cell having at least one inactivated  $\alpha$ -1,3 galactosyltransferase allele, where the totipotent cell 5 is derived from the aforementioned mammalian species having a functional  $\alpha$ -1,3 galactosyltransferase gene, manipulating the totipotent cell such that mitotic descendants of the cell constitute all or part of a developing embryo, allowing the embryo to develop to 10 term, recovering a neonate individual derived from the embryo, and raising and breeding the neonate to obtain a mammal homozygous for an inactivated  $\alpha$ -1,3 galactosyltransferase allele, i.e., a mammal in which both  $\alpha$ -1,3 galactosyltransferase allele are inactivated.

15 The totipotent cells can include, without limitation, ES cells, PGC's and eggs. The cells can be taken from a variety of mammalian species in which alleles for the  $\alpha$ -1,3 galactosyltransferase gene are present and functional, including without limitation 20 murine and porcine species. ES cells and PGC's are manipulated in various ways such that their mitotic descendants are found in a developing embryo. These manipulations can include, without limitation, injection into a blastocyst or morula, co-culture with a zona 25 pellucida-disrupted morula, and fusion with an enucleated zygote. Cells injected into a blastocyst- or morula-stage embryo become incorporated into the inner cell mass

of the blastocyst embryo, giving rise to various differentiated cell types of the resulting embryo, including in some cases germ cells. The embryo derived from such manipulations is a chimera composed of normal 5 embryonic cells as well as mitotic descendants of the introduced ES cells or PGC's. Alternatively, chimeric embryos can be obtained by co-culturing at least one ES cell or PGC with a morula embryo in which the zona pellucida is sufficiently disrupted to allow direct 10 contact between the ES cell/PGC and at least one cell of the morula. The zona pellucida-disrupted embryo may be an embryo that is completely free of the zona pellucida. Finally, the genome of an ES cell or PGC can be 15 incorporated into an embryo by fusing the ES cell/PGC with an enucleated zygote. Such a procedure is capable of generating a non-chimeric embryo, i.e., an embryo in which all nuclei are mitotic descendants of the fused ES cell/PGC nucleus. The resulting embryos are implanted in a recipient female, or surrogate mother, and allowed to 20 develop to term.

When eggs, as opposed to ES cells or PGC's, are directly injected with a nucleic acid construct effective for inactivating the  $\alpha$ -1,3 galactosyltransferase gene, the eggs can be manipulated to form an embryo by 25 implanting into a recipient female.

The invention also includes a mammal, produced through human intervention, that lacks a functional  $\alpha$ -1,3

galactosyltransferase gene. The mammal belongs to a species in which the  $\alpha$ -1,3 galactosyltransferase gene is normally present and functional. The mammal can be, without limitation, a mouse or a pig.

5 The invention further includes a purified and isolated nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of (1) the nucleic acid sequence depicted in Figure 26 (SEQ ID NO: 25), (2) a sequence corresponding to the sequence of (1) 10 within the scope of the degeneracy of the genetic code, and (3) a sequence that encodes murine T-LIF and that hybridizes under standard high stringency conditions with a sequence complementary to the sequence of (1) or (2). The complementary strands to the above-described nucleic 15 acid sequences are readily determined by standard methods, and are also within the scope of the present invention.

The present invention also includes a host cell transformed with any of the purified and isolated nucleic 20 acid molecules described in the preceding paragraph, as well as a T-LIF polypeptide encoded by such transforming nucleic acid molecules and expressed from the host cell.

The invention further includes a purified and isolated nucleic acid molecule comprising a nucleic acid 25 sequence selected from the group consisting of (1) the nucleic acid sequence depicted in Figure 27 (SEQ ID NO: 31), (2) a sequence corresponding to the sequence of (1)

within the scope of the degeneracy of the genetic code, and (3) a sequence that encodes human T-LIF and that hybridizes under standard high stringency conditions with a sequence complementary to the sequence of (1) or (2).

5 The complementary strands to the above-described nucleic acid sequences are readily determined by standard methods, and are also within the scope of the present invention.

The present invention also includes a host cell  
10 transformed with any of the purified and isolated nucleic acid molecules described in the preceding paragraph, as well as a T-LIF polypeptide encoded by such transforming nucleic acid molecules and expressed from the host cell.

The invention further includes a method for  
15 eliminating or reducing hyperacute rejection of non-primate mammalian cells by human serum, comprising adding, to the human serum, a physiologically acceptable amount of galactose or a saccharide in which the terminal carbohydrate is an  $\alpha$  galactose linked at position 1,  
20 prior to exposure of the human serum to the non-primate cells. The amount of galactose or saccharide added is sufficient to reduce or eliminate the hyperacute rejection response. The saccharide can be, without limitation, melibiose, galactose  $\alpha$ 1-3 galactose or  
25 stachyose. Alternatively, the human serum can be treated so as to be substantially depleted of immunoglobulin, IgM antibodies, anti-GAL IgM and IgG antibodies, or anti-GAL

IgM antibodies. The invention further includes affinity-treated human serum substantially free of anti-GAL antibodies or of anti-GAL IgM antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

5 FIGURE 1 is a graphical representation of fluorescence intensity following immunofluorescent staining of porcine aortic endothelial cells with anti-GAL antibody alone or with anti-GAL antibody that was preincubated with selected saccharides.

10 FIGURE 2 shows the results of an experiment in which lysis of porcine aortic endothelial cells by human serum and by purified anti-GAL antibodies was determined using a <sup>51</sup>CR release assay.

15 FIGURE 3 depicts physiograph tracings of perfused rat heart contractions in the presence of human serum with or without selected saccharides.

FIGURE 4 is a comparison of the porcine  $\alpha$ -1,3 galactosyltransferase cDNA sequence with the corresponding murine and bovine sequences. PGTCD =  
20 porcine sequence. BOVGSTA = bovine sequence. MUSGLYTNG = murine sequence.

FIGURE 5 is a comparison of the porcine  $\alpha$ -1,3 galactosyltransferase amino acid sequence with the corresponding murine and bovine amino acid sequences.  
25 PGT = porcine sequence. BGT = bovine sequence. MGT = murine sequence.

FIGURE 6 depicts the Sal1 restriction sites in four overlapping phage clones spanning a portion of the murine  $\alpha$ -1,3 galactosyltransferase genomic region.

FIGURE 7 is a detailed restriction map of murine 5  $\alpha$ -1,3 galactosyltransferase subclone p $\alpha$ GT-S5.5.

FIGURE 8 is a detailed restriction map of murine  $\alpha$ -1,3 galactosyltransferase subclone p $\alpha$ GT-S4.0.

FIGURE 9 is a detailed restriction map of murine  $\alpha$ -1,3 galactosyltransferase subclone p $\alpha$ GT-S11.

10 FIGURE 10 is a detailed restriction map of murine  $\alpha$ -1,3 galactosyltransferase subclone p $\alpha$ GT-S13.

FIGURE 11 is an additional detailed restriction map of murine  $\alpha$ -1,3 galactosyltransferase subclone p $\alpha$ GT-S5.5.

15 FIGURE 12 is an additional detailed restriction map of murine  $\alpha$ -1,3 galactosyltransferase subclone p $\alpha$ GT-S4.0.

FIGURE 13 is a diagram of a knockout construct carrying the 4.0 and 5.5kb Sal1 fragments from p $\alpha$ GT-S5.5 20 and p $\alpha$ GT-S4.0, which flank the Exon 9 Sal1 site.

FIGURE 14 depicts the 8.3kb and 6.4kb BglII fragments that are diagnostic for the uninterrupted  $\alpha$ -1,3 galactosyltransferase gene and the targeted (inactivated)  $\alpha$ -1,3 galactosyltransferase gene, respectively, using the 25 probes identified in the text.

FIGURE 15 is a schematic representation of the generation of a knockout construct using the vector p $\alpha$ GT-S5.5 as the starting vector.

FIGURE 16 sets out the nucleotide sequence of a 5 neomycin resistance cassette used in the construction of a DNA construct for interrupting the  $\alpha$ -1,3-GalT gene in mice.

FIGURE 17 is a diagram of one example of a final knockout construct that has been sequenced to confirm the 10 identity, copy number and orientation of the various inserts.

FIGURE 18 is a Southern blot of genomic DNA from various murine ES cell lines transformed with the knockout construct of Figure 16, probed to reveal the 15 diagnostic fragments depicted in Figure 14.

FIGURE 19 depicts the "long" PCR products derived from wild type and interrupted  $\alpha$ -1,3-GalT genes using the designated primers.

FIGURE 20 is a Southern blot of long PCR products 20 obtained from wild type and knockout mice.

FIGURE 21 depicts the PCR products used for identification of the interrupted (targeted) galt locus.

FIGURE 22 shows PCR products generated from mice carrying interrupted (inactivated) GalT alleles.

25 FIGURE 23 depicts the PCR products expected from PCR analysis of cDNA generated from  $\alpha$ -1,3-GalT mRNA in normal and knockout mice. The ferrochelatase primers and

PCR fragment represent a control demonstrating that cDNA synthesis had occurred.

FIGURE 24 shows the PCR fragments generated from cDNA obtained from RNA isolated from kidney (K), heart 5 (H) and liver (L) of a wild-type mouse (+/+) , a mouse heterozygous for the interrupted  $\alpha$ -1,3-GalT allele (+/-) and a mouse homozygous for the interrupted  $\alpha$ -1,3-GalT allele (-/-) .

FIGURE 25 is a graphical representation of the 10 relative protection of spleen cells, derived from GalT knockout mice, from lysis by human serum.

FIGURE 26 is a representation of the nucleotide sequence and deduced amino acid sequence for murine T-LIF.

15 FIGURE 27 is a representation of the nucleotide sequence and deduced amino acid sequence for human T-LIF.

FIGURE 28 is a Western blot of LIF polypeptides expressed from transfected COS cells.

20 FIGURE 29 is a diagram of the expression plasmid used for transfection of the COS cells of Figure 27.

FIGURE 30 is a Southern blot of PCR-amplified cDNA from murine ES cells, using a LIF-specific probe.

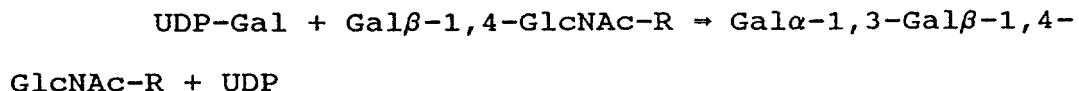
#### DETAILED DESCRIPTION

Evidence presented herein establishes that a 25 substantial portion of human pre-formed, anti-pig xenoantibodies recognize a specific terminal galactose

linkage on the surface of pig endothelial cells. As demonstrated in experiments carried out by the present inventors, it is possible to reduce the titers of preformed xenoantibodies by adsorption with immobilized 5 antigens containing the appropriate epitopes. This leads to reduction or elimination of cellular responses associated with the hyperacute rejection response. Conversely, it is demonstrated to be possible to neutralize such antibodies by addition of appropriate 10 carbohydrate antigens to human serum. In demonstrating the usefulness of these approaches, it was necessary to identify the relevant carbohydrate moieties and to demonstrate their efficacy in cultured cell systems and, importantly, in whole organs. As such, one approach to 15 reducing or eliminating the hyperacute rejection response is identified as treatment of the recipient by eliminating or neutralizing the relevant antibody populations.

An alternative approach to xenotransplantation 20 would be elimination of the relevant epitope(s) in the donor organ. This could be accomplished, for example, by reducing or eliminating expression of the gene(s) encoding the metabolic machinery responsible for formation of the epitopes. The epitope defined by the  $\alpha$ - 25 1,3 galactose linkage (termed the GAL epitope) is generated by the enzyme UDP-galactose: $\beta$ -D-galactosyl-1,4-N-acetyl-D-glucosaminide  $\alpha$ -1,3 galactosyl- transferase

(" $\alpha$ -1,3 galactosyltransferase" or " $\alpha$ -1,3-GalT"). This enzyme transfers galactose to the terminal galactose residue of N-acetyllactosamine-type carbohydrate chains and lactosaminoglycans. The reaction catalyzed by  $\alpha$ -1,3-GalT may be summarized as follows:



The  $\alpha$ -1,3-Gal T enzyme is found in most mammals, but is not present in Old World monkeys and humans. For 10 purposes of xenotransplantation, it is significant that humans and Old World monkeys have naturally occurring xenoantibodies directed against the GAL epitope. The use of pig organs lacking the GAL epitope could reduce or eliminate the hyperacute rejection of such organs by 15 human recipients. The utility of such an approach is buttressed by the present inventors' demonstration that the GAL epitope is, in fact, central to the hyperacute rejection phenomenon in cells and whole organs. One approach to obtaining such organs would be to generate 20 pigs in which the gene encoding the  $\alpha$ -1,3-GalT enzyme is "knocked out" by homologous recombination.

#### Role of the GAL Epitope in Hyperacute Rejection

The present inventors have affinity purified antibodies directed against the GAL epitope (anti-GAL 25 antibodies) from human serum. This was accomplished with affinity columns comprising the appropriate epitopes

(e.g., galactosyl-galactose or melibiose) attached to a solid phase. Total anti-GAL IgG and IgM were obtained in one set of experiments. In an alternative approach, anti-GAL IgG was obtained by passage of serum over an 5 affinity column with specificity for all proteins except albumin and IgG. The wash-through from this column was then applied to a galactosyl-galactose affinity column and purified anti-GAL IgG was collected as the eluate. The obtained anti-GAL IgG can be further purified by 10 passage over a protein G column, which specifically binds IgG but not other antibody isotypes. Conversely, the wash-through from the above-described columns can be used as sources of total anti-GAL (IgG + IgM)-depleted serum or of anti-GAL IgG-depleted serum in further experiments. 15 Preferably, the anti-GAL antibody preparations are characterized for protein content, molecular weight and purity, and for antibody class and isotype.

To demonstrate the role of the GAL epitope in the hyperacute rejection response, it is necessary, first, to 20 establish that IgG and IgM anti-GAL antibodies react with porcine cells and tissues. The present inventors investigated the binding of human anti-GAL antibodies to porcine cells and tissues using immunofluorescent staining. In this technique, selected human antibody 25 preparations are reacted with intact porcine cells and then reacted with signal antibody comprising non-human anti-human IgG or IgM labeled with fluorescein

isothiocyanate (FITC). Stained cells may be detected and quantified with a fluorescence-activated cell sorter (FACS) or other appropriate detection means. Other methods for detecting the presence of a bound antibody on 5 a cell surface, for example through use of enzyme-labeled signal antibody reagents, are known to the skilled artisan.

Total anti-GAL (IgM and IgG), as well as purified anti-GAL IgG, stained cells from a porcine epithelial 10 cell line (PK<sub>1</sub>) as well as cells from a porcine aortic endothelial cell line (PAE). Neither anti-GAL (total IgM + IgG) antibody-depleted serum nor anti-GAL IgG-depleted serum gave detectable staining. To further investigate the specificity of the response, it is desirable to 15 determine whether or not reactivity of the antibodies with porcine cells can be diminished or eliminated by prior exposure to one or more molecules suspected of comprising the epitope(s) in question. In this regard, the present inventors have established that antibody 20 binding is inhibited by galactose and by disaccharides having terminal galactose residues in the  $\alpha 1$  configuration. Staining was not inhibited with sugars having a terminal galactose in a  $\beta 1 \rightarrow 4$  configuration. These results demonstrate the specificity of the antibody 25 binding and the ability of appropriate sugars to inhibit such binding.

Reactivity of anti-GAL antibodies with cultured pig cells was confirmed using tissue sections of pig organs. Again, using a fluorescent signal antibody system, staining was seen with total anti-GAL IgM + IgG 5 and with purified anti-GAL IgG but not with the anti-GAL antibody-depleted sera. Staining was particularly strong with kidney, heart and liver endothelium, with heart endocardium and with bile duct epithelium. The tissue binding was inhibited with melibiose but was not 10 inhibited by other disaccharides not representative of the GAL epitope.

These data clearly indicate that the GAL epitope is expressed at high levels on the endothelial cells of arteries, veins and capillaries of porcine kidney, heart 15 and liver. In a xenograft situation, the endothelial cells of these vessels come into direct contact with the anti-GAL antibodies in human serum. The above results are consistent with evidence that binding of these antibodies (with attendant complement activation) is a 20 key component of the hyperacute rejection response.

To further investigate the specificities of naturally occurring xenoantibodies in human serum directed against porcine antigens, the ability of human serum to cause agglutination of pig red blood cells was 25 investigated. These studies revealed the presence of high levels of such antibodies in human serum. Moreover, sugars such as melibiose, stachyose, galactose and

fucose, having terminal residues in the  $\alpha$ 1-6 configuration, were found to inhibit agglutination in the  $\mu$ M to mM range. Sugars with other configurations were only inhibitory at very high doses, where the observed 5 effects are likely due to simple changes in osmolarity or other non-specific mechanisms.

The above investigations establish a potential role for naturally occurring, human anti-GAL xenoantibodies in the complement-mediated destruction 10 underlying hyperacute rejection. However, it is preferable to directly examine complement-mediated destruction of porcine cells in order to confirm the specificity of the GAL epitope and of anti-GAL antibodies in the process of lysis. To this end, the present 15 inventors have examined the ability of human serum to cause lysis of porcine cells.

To investigate complement-mediated destruction of cells, it is necessary to employ one or more assays that provide quantitative data on cell lysis. Preferably, 20 such assays measure a cell-sequestered component that is released into the medium upon complement-mediated cell lysis. Such experiments should control for involvement of complement in the induced lysis by employing both native complement proteins as well as heat-inactivated 25 complement. The present inventors have used a  $^{51}\text{Cr}$ -release assay and a lactate dehydrogenase (LDH)-release

assay to investigate the complement-mediated lysis of porcine epithelial and endothelial cells by human serum.

In the  $^{51}\text{Cr}$ -release assay, porcine cells were pre-labeled with  $^{51}\text{Cr}$  and then incubated in the presence of 5 heat-inactivated human serum plus rabbit complement (PAE's) or human complement in non-heat-inactivated normal human serum (PK<sub>1</sub>'s). Release of  $^{51}\text{Cr}$  into the medium was measured with a gamma counter following addition of scintillation fluid. In the LDH-release 10 assay, cells were labeled with LDH as per the manufacturer's instructions (Promega, USA). Release of LDH into the medium was measured using an ELISA format, with absorbance read at 492nm. For both assays, the ability of various sugars to inhibit the complement-15 induced lysis was also tested.

Similar results were obtained with the two unrelated porcine cell lines, PAE and PK<sub>1</sub>, using both types of assays. The results clearly demonstrate that naturally occurring xenoantibodies (NXAb's) are 20 responsible for initiating the complement-induced lysis of porcine cells. The present inventors have also established that IgM and not IgG antibodies are responsible for the lysis in this system. Moreover, heat inactivation of the complement preparations prevented 25 lysis, providing further evidence that lysis of the porcine cells is a complement-dependent phenomenon. The present inventors have also shown that melibiose, but not

lactose, protects the porcine cells from lysis.

Importantly, the concentrations of sugar found to be effective in these studies covered the physiological range of blood sugar, i.e., about 10mM.

5        These results indicate that the anti-GAL NXAb's in normal human serum are primarily responsible for lysis of the porcine cells. As such, the binding of anti-GAL NXAb's to the endothelial cells lining the blood vessels of a porcine xenograft, with attendant activation of the 10 complement cascade, is likely to be a key component of the hyperacute rejection of porcine xenografts. This would also be the case with organs from other discordant species, such as rodents, sheep, cows and goats, all of which have active  $\alpha$ -1,3-GaIT genes in their genomes.

15       These conclusions are further supported in a whole-organ study performed by the present inventors. For this study, isolated and perfused rat hearts were used to further demonstrate the involvement of anti-GAL xenoantibodies in hyperacute rejection. Rat hearts were 20 connected to a Langendorf perfusion apparatus, as described in Doring and Dehnert, The Isolated Perfused Heart According to Langendorf, Bionesttechnik-Verlag March GmbH, D7806, West Germany. The connected hearts were then stabilized by perfusion with a physiological 25 buffer system, and perfused with the same buffer containing either melibiose or lactose (10mM). Human plasma was then added to a final concentration of 13% and

the effect of the added sugar on heart rate, strength of contraction and output were measured.

These results demonstrate in a whole-organ system that:

5 1) Perfusion with unmodified human plasma causes rapid loss of function.

2) Perfusion of a rat heart with human plasma in the presence of melibiose, which competes for binding with the anti-GAL antibodies, prolongs heart survival and 10 output. Lactose, however, which does not compete for binding with the anti-GAL antibodies, does not prolong heart survival.

3) Perfusion of a rat heart with anti-GAL antibody-depleted plasma prolongs heart survival and 15 output.

4) If purified anti-GAL antibodies are added back to anti-GAL antibody-depleted plasma, the heart rapidly loses function

The present inventors' experiments with cultured 20 cells, tissues and whole organs provide important confirmation that anti-GAL antibodies are a critical element in the hyperacute rejection response. Moreover, the disclosed results point to various approaches that can be employed to eliminate or reduce the hyperacute 25 rejection of xenogeneic mammalian organs by humans.

For example, the intravenous administration of the specific disaccharide galactose  $\alpha$  1-3 galactose will block the naturally occurring anti-GAL antibodies of all classes and prevent them binding to their specific epitopes on the surface of the endothelial cells of the xenograft, thus preventing them from initiating or participating in hyperacute rejection. The present inventors' results indicate that the concentration of galactose  $\alpha$  1-3 galactose required to achieve this effect is in a physiologically tolerated range. The experiments also indicate that various other carbohydrates can be substituted for the specific disaccharide. These include the monosaccharide galactose and various other di-, tri- or tetra-saccharides in which there is a terminal  $\alpha$  galactose linked to the next sugar via position 1. These other sugars include, but are not limited to, melibiose and stachyose.

Likewise, prior to xenotransplantation, all or a substantial portion of total IgM (that is, IgM of all specificities) can be removed from the serum of the patient by extracorporeal immunoabsorption. Alternatively, anti-GAL antibodies of all classes can be removed by extracorporeal immunoabsorption. Most preferably, the patient's pre-formed natural anti-GAL IgM antibodies can be removed. In this way, many or most of the primary immunological agents of the hyperacute response are eliminated, resulting in reduction or

elimination of the response following  
xenotransplantation.

The  $\alpha$ -1,3-GalT Gene as a Target for Suppressing  
the GAL Epitope

5 The present inventors have succeeded in cloning  
the entire coding region of the porcine  $\alpha$ -1,3-GalT gene.  
This is desirable for full exploitation of the gene in  
genetic engineering of pigs for purposes of human  
xenotransplantation. Previous attempts to obtain the  
10 entire coding region of the porcine gene have, to the  
knowledge of the inventors, failed to generate the 5'  
coding regions. See, e.g., Dabkowski et al., Transplant.  
Proc. 25: 2921 (1993). The present inventors have  
employed a PCR-based approach to generate the full  
15 sequence. In designing the primers and experimental  
conditions required to obtain the 5' and 3' regions of  
the gene, the present inventors overcame significant  
theoretical and practical obstacles to success.

Primers were selected on the basis of careful  
20 analysis of published sequences for the murine, bovine  
and human  $\alpha$ -1,3-GalT genes, the only published sequences  
available for this purpose. The present inventors'  
analysis revealed that in the reported sequence of the  
bovine cDNA, exon 3 (which is in the 5' -untranslated  
25 region) is missing. This had not been reported in the  
literature. Thus, in order to find appropriate regions

for deriving useful primer sequences, the mouse and bovine sequences had to be realigned. Even with the appropriate realignment, however, only one island of about 20 base pairs (bp) in the 5' untranslated region 5 displayed the desired homology (19 out of 20 bp) for design of a PCR oligonucleotide. The fact that the 5' untranslated regions of the mouse and bovine genes do not seem substantially related even upon optimal alignment would not be considered unusual by the ordinary skilled 10 artisan. This is because the 5' untranslated regions are often not well conserved between species. As such, the natural inclination would be to perform a less-than-exhaustive analysis and to conclude that design of PCR oligonucleotides based on homology from this region was 15 unlikely to be successful.

In the downstream 3' -untranslated region, the homology is less than obvious again. Various insertions and deletions had to be made in order to obtain proper alignment of the mouse and bovine sequences. Moreover, 20 to obtain a region of appropriate homology for design of PCR oligonucleotides, it was necessary to select a region approximately 200 bp downstream of the stop codon. Finally, to get the 5' and 3' primers to work properly, the present inventors found it necessary to drop the 25 annealing temperature by 9°C. These technical and theoretical hurdles to successful use of a PCR-based

approach were overcome by the present inventors and allowed the entire coding sequence to be determined.

Analysis of the nucleotide sequence indicates that a counterpart to murine exon 3 in the 5' untranslated region is not found in the porcine gene. The porcine sequence is similar to the bovine sequence in this regard. Analysis of the amino acid sequence demonstrates that the structure of the porcine  $\alpha$ -1,3-GalT is similar to that of other glycosyltransferases, and in particular is closely related to bovine and murine  $\alpha$ -1,3-GalTs. In each of these enzymes a short cytoplasmic amino-terminal domain of about 6 residues precedes a hydrophobic membrane-anchoring domain (extending from residues 7 to 22). The stem region, which serves as a flexible tether, and the catalytic domain, which catalyses the synthesis of  $\alpha$ -1,3-GAL linkages, are located in the lumen of the Golgi and extend from amino acid 23 to the carboxyl terminus at amino acid 371. The precise boundary between the stem and catalytic domains is not well-defined.

Based on the suggested characteristics of the stem region, it appears to be the least conserved region and is rich in glycine and proline residues. Paulson and Colley, J. Biol. Chem. 264: 17615 (1989); Joziasse et al., J. Biol. Chem. 267: 5534 (1992). The stem/catalytic boundary may occur around amino acid 60.

To generate constructs for inactivating genes by homologous recombination, the gene is preferably

interrupted within an appropriate coding exon by insertion of an additional DNA fragment. Upon analysis of the full-length porcine nucleic acid sequence, the present inventors have identified exons 4, 7, 8 and 9 as 5 preferred locations for disruption of the gene by homologous recombination. However, identification of these exons as preferred sites should not be construed as limiting the scope of the present invention, as interruptions in exons 5 and 6 may be useful in 10 particular cell types or in situations where less-than-complete inhibition of  $\alpha$ -1,3-GalT gene expression is desired. Moreover, regulatory elements associated with the coding sequence may also present useful targets for inactivation.

15 In a preferred embodiment, a Sal1 site located within exon 9 of the mouse  $\alpha$ -1,3-GalT gene at codons 221-222 is chosen as the site for disruption of the murine coding sequence. For disruption of the porcine sequence, it is noted that the amino acids encoded by the 20 corresponding porcine nucleotides are conserved, although the Sal1 site is not. In a preferred embodiment for inactivation of the porcine gene, a Sal1 site is engineered into the corresponding location of the pig sequence for convenient construction of a knockout 25 sequence. Sal1 cuts only rarely in genomic DNA. Since multiple restriction sites can be a problem in manipulating large fragments of DNA, the presence of a

Sal1 site in the exon is very useful since it is not likely that other Sal1 sites will be present at other locations in the knockout constructs.

A gene coding for a selectable marker is generally used to interrupt the targeted exon site by homologous recombination. Preferably, the selectable marker is flanked by sequences homologous to the sequences flanking the desired insertion site. Thomas and Capecchi, Cell 51: 503-12 (1987); Capecchi, Trends in Genetics 5: 70-76 (1989). It is not necessary for the flanking sequences to be immediately adjacent to the desired insertion site. The gene imparting resistance to the antibiotic G418 (a neomycin derivative) frequently is used, although other antibiotic resistance markers (e.g., hygromycin) also may be employed. Other selection systems include negative-selection markers such as the thymidine kinase (TK) gene from herpes simplex. Any selectable marker suitable for inclusion in a knockout vector is within the scope of the present invention.

However, it is possible that in some circumstances it will not be desirable to have an expressed antibiotic resistance gene incorporated into the cells of a transplanted organ. Therefore, in a preferred embodiment, one or more genetic elements are included in the knockout construct that permit the antibiotic resistance gene to be excised once the construct has

undergone homologous recombination with the  $\alpha$ -1,3-GalT gene.

The FLP/FRT recombinase system from yeast represents one such set of genetic elements. O' Gorman et al., Science 251, 1351-1355 (1991). FLP recombinase is a protein of approximately 45 kD molecular weight. It is encoded by the FLP gene of the 2 micron plasmid of the yeast *Saccharomyces cerevisiae*. The protein acts by binding to the FLP Recombinase Target site, or FRT; the core region of the FRT is a DNA sequence of approximately 34 bp. FLP can mediate several kinds of recombination reactions including excision, insertion and inversion, depending on the relative orientations of flanking FRT sites. If a region of DNA is flanked by direct repeats of the FRT, FLP will act to excise the intervening DNA, leaving only a single FRT. FLP has been shown to function in a wide range of systems, including in the cultured mammalian cell lines CV-1 and F9, O' Gorman et al., Science 251: 1351 (1991), and in mouse ES cells, Jung et al., Science 259: 984 (1993).

Targeted cells carrying a genomic copy of an antibiotic resistance gene flanked by direct repeats of the FRT are supplied with FLP recombinase by 1) introduction into cells of partially purified FLP protein by electroporation, or 2) transfection with expression plasmids containing the FLP gene. In this way, the antibiotic resistance gene is deleted by action of the

FLP recombinase, and cells are generated that contain the inactivated  $\alpha$ -1,3-GalT gene and are free of the exogenous antibiotic resistance gene.

Due to the relative infrequency of homologous recombination in targeted cells, most such cells will carry only one inactivated allele of the target gene. That is, the great majority of cells taken through a single round of transformation with an appropriate knockout construct will be heterozygotes. As used herein, the term "transformed" is defined as introduction of exogenous DNA into the target cell by any means known to the skilled artisan. These methods of introduction can include, without limitation, transfection, microinjection, infection (with, for example, retroviral-based vectors), electroporation and microballistics. The term "transformed," unless otherwise indicated, is not intended herein to indicate alterations in cell behavior and growth patterns accompanying immortalization, density-independent growth, malignant transformation or similar acquired states in culture.

Although heterozygous cells can be used in the methods of the present invention, various manipulations can be employed to generate homozygous cells in culture. For example, homozygous cells can be generated by performing a second homologous recombination procedure on cells heterozygous for the inactivated allele. If the knockout construct used in the initial transformation

carried the neo<sup>R</sup> gene, a second construct may be employed in a second round of transformation in which the neo<sup>R</sup> gene is replaced with a gene conferring resistance to a separate antibiotic (e.g., hygromycin). Cells resistant 5 to both G418 and hygromycin can be screened by Southern blots in order to detect any "double knockouts" (i.e., homozygotes). Both antibiotic resistance genes can be removed subsequently in a single procedure using FLP recombinase. By maintaining selection with G418, this 10 approach ensures that the second construct does not simply replace the previously knocked-out allele, leaving the cells heterozygous.

Alternatively, the neo<sup>R</sup> gene can be deleted from an original heterozygous cell using FLP recombinase and a 15 second knockout procedure conducted using the original neo<sup>R</sup> gene-containing construct. Double knockouts could be detected by Southern analysis. The newly introduced neo<sup>R</sup> gene then could be deleted by FLP recombinase. This alternative approach does not allow one to direct the 20 knockout construct specifically to the non-inactivated allele. Nevertheless, screening of appropriate numbers of targeted cells can lead to identification of cells homozygous for the inactivated locus.

Cellular Vehicles for Incorporation of Knockout Constructs

25 To create animals having a particular gene inactivated in all cells, it is necessary to introduce a

knockout construct into the germ cells (sperm or eggs, i.e., the "germ line") of the desired species. Genes or other DNA sequences can be introduced into the pronuclei of fertilized eggs by microinjection. Following 5 pronuclear fusion, the developing embryo may carry the introduced gene in all its somatic and germ cells since the zygote is the mitotic progenitor of all cells in the embryo. Since targeted insertion of a knockout construct is a relatively rare event, it is desirable to generate 10 and screen a large number of animals when employing such an approach. Because of this, it can be advantageous to work with the large cell populations and selection criteria that are characteristic of cultured cell systems. However, for production of knockout animals 15 from an initial population of cultured cells, it is necessary that a cultured cell containing the desired knockout construct be capable of generating a whole animal. This is generally accomplished by placing the cell into a developing embryo environment of some sort.

20 Cells capable of giving rise to at least several differentiated cell types are hereinafter termed "pluripotent" cells. Pluripotent cells capable of giving rise to all cell types of an embryo, including germ cells, are hereinafter termed "totipotent" cells. 25 Totipotent murine cell lines (embryonic stem, or "ES" cells) have been isolated by culture of cells derived from very young embryos (blastocysts). Such cells are

capable, upon incorporation into an embryo, of differentiating into all cell types, including germ cells, and can be employed to generate animals lacking a functional  $\alpha$ -1,3-GaIT gene. That is, cultured ES cells 5 can be transformed with a knockout construct and cells selected in which the  $\alpha$ -1,3-GaIT gene is inactivated through insertion of the construct within, for example, an appropriate exon. In fact, ES cell lines have been derived for both mice and pigs. See, e.g., Robertson, 10 Embryo-Derived Stem Cell Lines. In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach (E.J. Robertson, ed.), IRL Press, Oxford (1987); PCT Publication No. WO/90/03432; PCT Publication No. 94/26884. Generally these cells lines must be propagated 15 in a medium containing a differentiation-inhibiting factor (DIF) to prevent spontaneous differentiation and loss of mitotic capability. Leukemia Inhibitory Factor (LIF) is particularly useful as a DIF. Other DIF's useful for prevention of ES cell differentiation include, 20 without limitation, Oncostatin M (Gearing and Bruce, The New Biologist 4: 61-65 (1992); personal communication from A. Smith), interleukin 6 (IL-6) with soluble IL-6 receptor (sIL-6R) (Taga et al., Cell 58: 573-81 (1989); personal communication from A. Smith), and ciliary 25 neurotropic factor (CNTF) (Conover et al., Development 19: 559-65 (1993). Other known cytokines may also

function as appropriate DIF's, alone or in combination with other DIF's.

As a useful advance in maintenance of ES cells in an undifferentiated state, the present inventors have 5 identified a novel variant of LIF. In contrast to the previously identified forms of LIF which are extracellular, this new form of LIF (hereinafter T-LIF) is intracellularly localized. The transcript was cloned from murine ES cells using the RACE technique, Frohman et 10 al., Proc. Natl. Acad. Sci. USA 85: 8998-9002 (1988), and subjected to sequence analysis. Analysis of the obtained nucleic acid sequence and deduced amino acid sequence indicates that T-LIF is a truncated form of the LIF sequence previously reported in the literature. 15 Expression of the T-LIF nucleic acid in an appropriate host cell yields a 17 kD protein that is unglycosylated. This protein is useful for inhibiting differentiation of murine ES cells in culture. The protein is expected to have a similar activity with porcine cells, since murine 20 D-LIF is effective at inhibiting both murine and porcine ES cell differentiation. The present inventors have also determined the sequence of the human form of T-LIF.

To generate a knockout animal, ES cells having at least one inactivated  $\alpha$ -1,3-GalT allele are identified 25 and incorporated into a developing embryo. This can be accomplished through injection into the blastocyst cavity of a murine blastocyst-stage embryo, by injection into a

morula-stage embryo, by co-culture of ES cells with a morula-stage embryo, or through fusion of the ES cell with an enucleated zygote. The resulting embryo is raised to sexual maturity and bred in order to obtain 5 animals, all of whose cells (including germ cells) carry the inactivated  $\alpha$ -1,3-GalT allele. If the original ES cell was heterozygous for the inactivated  $\alpha$ -1,3-GalT allele, several of these animals must be bred with each other in order to generate animals homozygous for the 10 inactivated allele.

Although direct microinjection of DNA into eggs does not generate the large numbers of recombination events obtained through transfecting large numbers of cultured cells, nevertheless direct injection of eggs can 15 be a useful approach since this avoids the special manipulations (see above) required to turn a cultured cell into an animal. This is because fertilized eggs are, of course, quintessentially "totipotent" - i.e., capable of developing into an adult without further 20 substantive manipulation other than implantation into a surrogate mother. To enhance the probability of homologous recombination when eggs are directly injected with knockout constructs, it is useful to incorporate at least 25 about 8 kb of homologous DNA into the targeting construct. In addition, it is also useful to prepare the knockout constructs from isogenic DNA. For example, for

injection of porcine eggs, it is useful to prepare the constructs from DNA isolated from the boar whose sperm are employed to fertilize the eggs used for injection.

Embryos derived from microinjected eggs can be  
5 screened for homologous recombination events in several ways. For example, if the GalT gene is interrupted by a coding region that produces a detectable (e.g., fluorescent) gene product, then the injected eggs are cultured to the blastocyst stage and analyzed for  
10 presence of the indicator polypeptide. Embryos with fluorescing cells, for example, are then implanted into a surrogate mother and allowed to develop to term. Alternatively, injected eggs are allowed to develop and the resulting piglets analyzed by polymerase chain  
15 reaction (PCR) or reverse transcription PCR (RT/PCR) for evidence of homologous recombination.

#### Characterization of Knockout Animals

Animals having either one (heterozygous) or two (homozygous) inactivated GalT genes are characterized to  
20 confirm the expected alterations in gene expression and phenotypic effect. For example, GalT mRNA should be absent from homozygous knockout animals. This can be confirmed, for example, with reverse transcription PCR (RT-PCR) using appropriate GalT-specific primers. In  
25 addition, various tests can be performed to evaluate expression of the GAL epitope in homozygous knockout animals. For example, anti-GAL antibodies and IB<sub>4</sub> Lectin

(which has an exclusive affinity for terminal  $\alpha$ -D-galactosyl residues) can be used in various assay or immunohistological formats to test for the presence of the GAL epitope in an array of tissues. As another 5 indication of GAL epitope status, lysis of cells by human serum can be tested through use of a  $^{51}\text{Cr}$  chromium release assay.

EXAMPLE 1

Affinity Purification of Human Anti-GAL Antibodies

10 Anti-GAL antibodies were purified from normal heat inactivated AB serum (from CS1, Parkville, Victoria, Australia) using the following sets of procedures.

A. Preparation of total anti-GAL (IgG+IgM) antibodies

The following procedures are performed at 4°C.

15 1. Desalt 15-30ml serum (in 3ml batches) by passage through a pre-equilibrated (20ml application buffer: 20mM  $\text{K}_2\text{HPO}_4$ , 30mM NaCl, pH 8) Econo Pac 10DG (Bio-Rad, Richmond, USA) column. Alternatively, for large scale preparations, desalt by dialysis exhaustively against 20 application buffer.

2. Wash column with 4ml aliquots of application buffer. Collect and pool column eluates.

3. Apply pooled desalting serum to a pre-equilibrated (20ml application buffer) Synsorb 115 (galactosyl-25 galactose; Chembomed, Alberta, Canada) or D(+) Melibiose-Agarose (Sigma) affinity column (5ml-50 ml depending on the yield required).

4. Collect run-through (partially anti-GAL-depleted) and reapply to column. Repeat process 3 times to ensure 30 complete removal of anti-GAL antibodies. The wash-through from the 3rd passage through the Synsorb column is collected and the volume adjusted to the original volume of the serum with phosphate-buffered saline (PBS)

pH 7 +0.05% azide. This is used as a source of anti-GAL antibody-depleted serum.

5. Wash column with PBS pH 8 until the eluate is protein free (O.D. 280nm=0).

5 6. Elute anti-GAL antibodies with 3.5M KSCN, pH 7.5. Collect 4ml fractions, determine the O.D. 280 and pool peak fractions (usually 1-6).

7. Concentrate anti-GAL antibodies using CF25 ultrafiltration cones (Amicon, Danvers, USA). Add 7ml of 10 the pooled fractions containing anti-GAL antibodies to spin cone and centrifuge (2,000 RPM, 10min, 4°C). Refill cone and recentrifuge until volume is reduced to 3-5ml.

8. To dilute the KSCN, adjust vol. to 7ml with PBS and centrifuge (2,000 RPM, 10min, 4°C). Repeat process a 15 further 10 times.

9. Remove sample containing anti-GAL antibodies from cone using plastic pipette; rinse cone with PBS pH7 +0.05% azide.

B. Preparation of IgG anti-GAL antibodies

20 The following procedures are performed at 4°C.

1. Desalt 15-30 ml serum (in 3ml batches) by passage through a pre-equilibrated (20ml application buffer) Econo Pac 10DG (Bio-Rad, Richmond, USA) column.

Alternatively for large scale preparations desalt by 25 dialysis exhaustively against application buffer.

2. Wash column with 4ml aliquots of application buffer. Collect and pool column eluates.

3. Apply desalting serum to a pre-equilibrated (30ml application buffer) Affi-Blue column (Bio-Rad, Richmond, 30 USA) (Affi-Blue binds all proteins except albumin and IgG).

4. Wash column with 20ml application buffer to elute IgG enriched fraction.

5. Apply IgG enriched fraction to a pre-equilibrated 35 (20ml application buffer, pH 8.0) Synsorb 115

(galactosyl-galactose; ChembioMed, Alberta, Canada) affinity column (5ml).

6. Collect run-through and reapply to column. Repeat process 3 times to ensure complete removal of anti-GAL antibodies. The wash-through from the 3rd passage through the Synsorb column is collected and the volume adjusted to the original volume of the serum with PBS pH 7 +0.05% azide. This is used as a source of control anti-GAL-depleted IgG.

10 In some cases anti-GAL IgG was further purified using a protein G column, which efficiently binds IgG but not other antibody isotypes. IgG was then eluted from the protein G column using glycine pH 2.4.

All anti-GAL antibody preparations were analyzed  
15 for the following:

20 a. Protein content was determined using the Bradford colorimetric method (Bradford, M.M 1976, Anal. Biochem. 72:248-254), using purified human IgG as the standard.

25 b. Molecular weight and purity were determined using polyacrylamide gel electrophoresis according to method described by Laemli, Nature (London) 227: 680 (1970), and protein was detected in the gels by silver staining using standard kit reagents (Amersham, UK).

30 c. Antibody class and isotype were determined by radial immunodiffusion using standard techniques as set out in Rose et al. (eds.), Manual of Clinical

5

Laboratory Immunology, American  
Society for Microbiology,  
Washington, D.C. IgG anti-GAL  
preparations were found to  
contain all subclasses, with  
IgG2 predominating.

EXAMPLE 2

Reactivity of IgG and IgM Anti-GAL Antibodies and  
Depleted Serum with Porcine Cells and Tissues

10 I. CELLS

Reactivity of IgG and IgM anti-GAL antibodies was assessed using either porcine aortic endothelial cells (prepared by the inventors as described below) or porcine epithelial cell line LLC PK<sub>1</sub> (PK<sub>1</sub>), obtained from the 15 American Type Culture Collection (ATCC), Accession No. CRL1392.

A. Isolation and culture of porcine aortic endothelial cells (PAE's)

Pigs were blood typed (using human typing 20 reagents) to identify "O-type" pigs, i.e., pigs unreactive with antibodies to A or B human red blood cell antigens. Aortas were excised from "O-type" pigs, then transported from the abattoir to the laboratory on ice. PAE's were isolated by collagenase treatment as described by 25 Gimbrone et al., J. Cell Biol. 60: 673-84 (1974). PAE's were cultured in RPMI medium containing 10% fetal calf serum (FCS), supplemented with 150 $\mu$ g/ml endothelial cell supplement (Sigma) and 50 $\mu$ g/ml heparin (Sigma). The cells were identified as endothelial cells by their 30 typical cobblestone morphology and by their immunoreactivity with Factor VIII antibodies, as identified using immunofluorescence. In all the assays

described below, the PAE's were used between the 8th and 12th passages.

B. Tissue Culture: Maintenance of PK-1 and PAE cell lines

5 All tissue culture was performed in a laminar flow hood, using appropriate tissue culture sterile technique. All tissue culture reagents, unless otherwise indicated, were purchased from CSL, Melbourne, Australia. Media were constituted as follows:

10 PK-1 Culture Medium:

DMEM (Cytosystems, Castle Hill, Australia)	500ml
FCS (CSL, Melbourne, Australia)	37.5ml
Glutamine (200mM) (Cytosystems)	5ml
Hepes (1M) (CSL)	7.5ml
15 Penicillin (CSL)	0.5ml ( $10^5$ U/ml final)
Streptomycin (CSL)	0.5ml ( $10^5$ $\mu$ g/ml final)

PAE - Culture Medium:

20	RPMI (CSL)	90ml
	FCS (CSL)	10ml
	Endothelial cell supplement (3mg/ml) (Sigma)	1.5ml
	Heparin (10mg/ml) (CSL)	0.5ml

Endothelial cell supplement was purchased from Sigma Chem. Co. (St. Louis, Missouri, USA) as a 25 lyophilized powder, resuspended in sterile HBBS, and 3ml aliquots stored at 4°C.

25	Heparin (Sigma, Missouri, USA) (10mg/ml)	- dissolved in PBS
30	(0.22 $\mu$ m)	- filter sterilized
	Hanks Buffer	- purchased from
	Cytosystems	

The following general procedures were used in propagating the cell lines.

35 1) Pour off old medium  
2) Rinse cells twice with sterile PBS

3) Add 3ml of TED (0.05 M trypsin, 0.53 M EDTA, Gibco, NY, USA)

4) Incubate 10 min. in CO<sub>2</sub> incubator at 37°C

5) Add 7ml RPMI with 10% FCS

6) Resuspend cells and transfer to a sterile 10ml tube

7) Centrifuge for 5min at 1200 rpm, discard supernatant

8) Resuspend cells in RPMI with 10% Newborn Bovine Serum (NBS) and repeat centrifugation

10) Resuspend cells in 1ml DMEM (PK-1's) or RPMI (PAE's) (with additives, as described above).

15) Add 10ml medium and the appropriate volume of cell suspension to achieve the desired dilution for each 75cm<sup>2</sup> tissue culture flask, and return to humidified CO<sub>2</sub> incubator.

C. Antibody staining and FACS analysis

20) 1) Add 2ml TED to a 75cm<sup>2</sup> culture flask containing PK-1 or PAE's, and incubate at room temperature for 10 min.

25) 2) Add RPMI plus 10% FCS (5ml) to neutralize trypsin.

3) Pellet cells by centrifugation (700g, 5 min, 4°C).

4) Wash cells by resuspension and centrifugation in Hanks Buffer (x2).

30) 5) Pellet cells by centrifugation (700g, 5 min, 4°C).

6) Resuspend cell pellet in Hanks buffer containing purified anti-GAL antibodies, GAL-depleted serum or GAL-depleted IgG and incubate at 4°C for 60 min. All

antibodies were used undiluted, or diluted 1:2 or 1:4 in Hanks buffer.

- 5 7) Add 1ml of Hanks Buffer, pellet cells by centrifugation and aspirate off supernatant.
- 10 8) Resuspend pellet in FITC-labelled sheep-anti-human IgG Fab2 or IgM Fab2 (Silenus, Hawthorn, Australia) diluted 1:80 in Hanks buffer.
- 15 9) Incubate for 30 min. at 4°C.
- 10 10) Wash three times with Hanks buffer; resuspend pellet from final wash in 0.5ml Hanks buffer.
- 11) Analyze stained samples using a FACScan II (Becton Dickinson) according to the manufacturer's instructions.

The specificity of the anti-GAL antibody binding to porcine cells was determined by examining the ability 20 of sugars of various structures to inhibit antibody binding. In these competition studies the anti-GAL antibodies were pre-incubated with sugar (0.1M) at 37°C for 30 min before adding to the cells.

D. Results

25 Using immunofluorescence it was found that total anti-GAL (IgM & IgG) and purified anti-GAL IgG stained both PK-1 and PAE's cells. On the other hand, neither the total anti-GAL antibody-depleted serum nor the anti-GAL IgG-depleted serum gave detectable staining over 30 background. The staining with anti-GAL IgM and/or IgG was inhibited with purified galactose and with disaccharides having terminal galactose residues in the  $\alpha$ 1-configuration such as melibiose (6-O- $\alpha$ -D-galactopyranosyl- D-glucose) and stachyose ( $\alpha$ -D-Gal-[1-

>6]- $\alpha$ -D-Glc-[1 $\rightarrow$ 2]- $\beta$ -D-Fru). Staining was not inhibited with sugars such as lactose (4-O- $\beta$ -D-galatopyranosyl- $\alpha$ -D-glucose), which has a terminal galactose residue, but in a  $\beta$ 1 $\rightarrow$ 4 configuration. The results of one such 5 experiment are represented in Figure 1. PAE's were stained with anti-GAL antibody alone (GAL:PBS) or with anti-GAL antibody that had been pre-incubated with either melibiose (GAL:MELIBIOSE), galactose (GAL:GALACTOSE) or lactose (GAL:LACTOSE). Anti-GAL antibody staining was 10 approximately 10 fold less in the samples containing melibiose and galactose, but was not affected significantly by lactose.

## II. TISSUES

### A. Methods

15 Pig kidney was fixed in formalin and dehydrated before embedding in Paraplast. Pig heart and liver were fixed in paraformaldehyde-lysine-periodate fixative and snap frozen in O.C.T. embedding compound (10.24% w/w polyvinyl alcohol, 4.26% w/w polyethylene glycol, 85.50% 20 w/w nonreactive ingredients; Tissue Tek®, Miles, Inc., Elkhart, Indiana, USA). Four  $\mu$ m-thick sections of pig heart and liver and 2  $\mu$ m-thick sections of kidney were incubated with purified anti-GAL antibodies (undiluted, 1:2 and 1:4) for 60 min. and then incubated with a 25 fluorescein isothiocyanate (FITC)-conjugated sheep anti-human immunoglobulin F(ab') fragment (Silenus Laboratories, Hawthorn, Australia) (1:100) for 30 min. or a peroxidase-conjugated rabbit anti-human IgG (Dakopatts, Glostrup, Denmark) (1:50) for 60 min. Control sections 30 were analyzed for autofluorescence, with the secondary antibody alone, or with the anti-GAL-depleted IgG or normal pig serum as the primary antibody. No staining was detected. The specificity of the anti-GAL antibodies was tested by pre-incubating sections of pig renal cortex

with a variety of sugars, including melibiose, lactose, sucrose and glucose at 0.1M.

B. Results

As with the analyses performed on the pig cells 5 using immunofluorescence, total anti-GAL IgM + IgG, purified anti-GAL IgG, but not the anti-GAL IgM and/or IgG-depleted sera, stained all pig tissues examined. The individual staining parameters varied from organ to organ as set out below:

10 Immunostaining of Pig Tissues with Anti-GAL Antibodies:

	<u>Tissue</u>	<u>Anti-GAL Reactivity</u>	<u>Staining Intensity</u>
15	Kidney	Proximal and distal convoluted tubules Endothelium: Intertubular sinusoids Endothelium: Arteries and veins Glomerular capillaries	Variable Variable Strong Variable
	Heart	Endothelium: Arteries, veins, capillaries Endocardium Myocardium	Strong Strong Perinuclear
	20 Liver	Small Bile Ducts (lining cells) Endothelium: Arteries, veins Intertubular sinusoids	Strong Strong Negative

The specificity of the binding of anti-GAL antibodies was tested on sections of pig renal cortex by inhibition with 0.1 M melibiose, lactose, sucrose and 25 glucose. Reactivity of the anti-GAL antibodies with proximal tubule brush borders was reduced to near background by preincubation of antibody with melibiose, but was not inhibited by the other saccharides. ... ..

EXAMPLE 3

30 Hemagglutination of Pig RBC by Human Serum:  
Sugar Inhibition Studies

The methods used to investigate the hemagglutination of pig red blood cells (RBC's) by human serum was adapted from the methods described by Galili,

J. Exp. Med. 160: 1579-81 (1984) and Severson, Immunol. 96: 785-789 (1966).

I. METHODS

A. Media/Solution Preparation

5 1. Human Serum Albumin (HSA) (CSL, Melbourne, Australia) (5mg/ml) was dissolved in PBS, filter sterilized, and stored at 4°C.

10 2. Preparation of sugars:

- 1M stock solutions of sugar were prepared by dissolving the amount indicated in 100ml of PBS. Sodium azide was added (0.02%) and solutions stored at 4°C.

15	$\alpha$ -Lactose (4-O- $\beta$ -D-galactopyranosyl- $\alpha$ -D-glucose D(+)-galactose	36.0g 18.0g
	Stachyose ( $\alpha$ -D-gal-[1->6]- $\alpha$ -D-Glc-[1->2]- $\beta$ -D-Fru)	66.6g
	Melibiose (6-O- $\alpha$ -D-galactopyranosyl- D-glucose) Sucrose ( $\alpha$ -D-Glucopyranosyl $\beta$ -D-fructofuranoside) D-(+)-Glucose	34.2 g 34.2 g 18.0 g
20	$\alpha$ -D-(+)-Fucose (6-Deoxy-D-galactopyranose)	16.4 g

All sugars were purchased from Sigma (St. Louis, Missouri, USA). Sugar solutions were diluted in PBS to the appropriate concentration as required.

B. Preparation of pig RBC's

25 1. Heparinised pig blood (Animal Resources, Clayton, Australia) is centrifuged at 800 RPM for 10min to pellet the RBC.

30 2. The RBC pellet is washed by resuspension in PBS (10ml) and recentrifugation (repeated 3 times). After the final wash, the RBC pellet is resuspended in 10ml PBS.

3. A 0.5% solution of RBC's is prepared by adding 50ul RBC solution (from step 2, above) to 10 ml PBS containing 0.5g/100 ml of HSA.

5

C. Preparation of 96-well microtitre plates  
(Titretek, USA)

10 1. Add 25ul of PBS to each well.

2. Add 25ul of pooled human AB serum (CSL, Melbourne, Australia) to column 1 and serially dilute by removing 25ul from column 1 and adding to column 2, then repeating by sequentially removing and adding 25ul from and to each well across the plate, finally discarding 25ul from column 11 and adding no serum to column 12.

15

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3. Add 25ul of sugar solution to each row in decreasing concentrations down rows. No sugar solution is added to the final row.

4. Incubate at 4°C overnight and then at 37°C for 30 min.

5. Add 50ul of 0.5% pig RBC to each well; vortex and incubate at room temperature for 2 hours. Determine agglutination visually.

### III. RESULTS

Human serum caused the agglutination of pig RBC's at a titre of between 1/32-1/64, which is consistent with the presence of high levels of naturally occurring xenoantibody (NXAb) in human serum. To examine the specificity of the NXAb response, sugar inhibition studies were performed. Sugars such as melibiose, stachyose, galactose and fucose which have terminal galactose residues in the  $\alpha$ 1-6 configuration were found to inhibit agglutination in the  $\mu$ M to mM range. Sugars with other structures, such as lactose and sucrose, were only inhibitory when very high concentrations were used. At these high concentrations, the observed effects are most probably non-specific, due, for example, to changes in osmolarity. Results are summarized below:

#### Pig RBC Hemagglutination by Human Serum: Sugar Inhibition

	<u>Sugar</u>	<u>Linkage</u>	<u>Inhibitory Concentration</u>
20	Melibiose	Gal $\alpha$ 1-6Glc	$5 \times 10^{-4}$ M
	Stachyose	Gal $\alpha$ 1-6Gal	$2 \times 10^{-3}$ M
	Galactose		$2 \times 10^{-3}$ M
	Fucose	6-Deoxy- $\alpha$ -L-Gal	$1 \times 10^{-3}$ M
	Lactose	Gal $\beta$ 1-4-Glc	$> 10^{-1}$ M
	Sucrose	$\alpha$ -D-Glc- $\beta$ -D-Fruc	$> 10^{-1}$ M

#### EXAMPLE 4

##### 25 Inhibition of Human Serum-Induced Lysis of Porcine Cells by Sugars

The ability of human serum to cause the lysis of porcine cells was examined using both pig epithelial (PK<sub>1</sub>) and aortic endothelial (PAE's) cells, the isolation and culture of which is described in Example 2. Cell lysis was determined using either the <sup>51</sup>Chromium release assay as described by Cerottini and Brunner, Nature New Biol. 237:272, 1972 or the Cytotox LDH release assay according to the manufacturer's instructions (Promega, USA).

I. METHODS

A. <sup>51</sup> CR Release Assay

1. Preparation of Cells:

a) Trypsinize a confluent flask of cells.

5 On average, approximately  $3 \times 10^6$  PAE's and approximately  $3 \times 10^7$  PK<sub>1</sub> cells are obtained per 10 ml flask. About  $1 \times 10^5$  cells are required for each well in the <sup>51</sup>CR Release Assay.

10 b) Wash cells 4 times in 10 ml RPMI (no FCS); spin 1200 rpm for 5 min.

c) Resuspend cells in 100  $\mu$ l RPMI (with 10% heat-inactivated FCS; see below).

2. Labelling Cells with <sup>51</sup> CR:

15 a) Combine in a 10 ml tube: Cells in 195  $\mu$ l RPMI/10% FCS (heat inactivated); 5  $\mu$ l <sup>51</sup> CR (120  $\mu$ Ci), b) Incubate at 37°C for 2 hr.

c) Add 2 ml RPMI/10% FCS (heat inactivated).

20 d) Centrifuge cells through a layer of FCS (heat inactivated) to remove excess label.

e) Gently overlay the labelled cells onto a 4 ml cushion of FCS using a Pasteur pipette.

f) Centrifuge at 700g for 5 min. at 4°C.

25 g) Remove supernatant taking care not to disturb the cell pellet.

h) Resuspend pellet in RPMI/10% FCS (heat inactivated) at about  $3 \times 10^7$  cells/ml.

3. Assay Conditions:

30 a) For PAE's, rabbit complement was used as the complement source, since the <sup>51</sup> CR-release assay was not sufficiently sensitive to detect lysis when human complement, a less "active" source, was used. In contrast, with the LDH assay, which is significantly more sensitive, normal human serum (NHS) was used as the 35 source of complement.

b) To each test well of a 96-well V bottom plate, add:

5 of final)

- 100  $\mu$ l labelled cells
- 10-50  $\mu$ l NHS (heat inactivated) (5-25% of final)
- Complement:
  - PAE's: 50  $\mu$ l absorbed rabbit complement (25% final)
  - PK<sub>1</sub>: 10-40  $\mu$ l NHS (5-25% of final)
- 50  $\mu$ l antibody (total anti-GAL (IgG + IgM, anti-GAL IgG, anti-GAL antibody-depleted serum, or anti-GAL antibody-depleted IgG)

c) Adjust volume to 200  $\mu$ l with

15 RPMI/10% FCS (heat inactivated) if required

d) Incubate plates at 37°C for 3 hr.

e) Centrifuge plates at 1000 rpm for 5 min to pellet cells

f) Remove 100  $\mu$ l of supernatant from

20 each well and transfer to a gamma counter tube

g) Add 3 ml scintillation fluid and measure <sup>51</sup>CR release using a gamma counter (Packard Instrument Company, Illinois, USA)

(To determine maximum release, add 100  $\mu$ l 8% Triton X-100 made up in RPMI/10% FCS (heat inactivated) to 100  $\mu$ l labelled cells)

(Note: Each reaction is set up in quadruplicate)

4. Calculation of % Lysis:

30 % Lysis =  $\frac{\text{Experimental cpm} - \text{Spontaneous Release cpm}}{\text{Max. Release cpm} - \text{Spontaneous Release cpm}} \times 100$

5. Sugar Inhibition of Complement-Induced Cell Cytotoxicity:

35 In a 96-well test plate, mix the following:

- 50  $\mu$ l labelled cells
- 50  $\mu$ l complement (PAE's: pig spleen cell absorbed complement; PK<sub>1</sub>'s: NHS)

10<sup>-1</sup> to 10<sup>-3</sup> M) - x  $\mu$ l sugar (final concentration of sugar:  
concentration 5-20%) - y  $\mu$ l NHS (heat inactivated) - final  
5 - make volume to 200  $\mu$ l with RPMI

Plate Layout:

	<u>Plate 1</u>		<u>Plate 2</u>	
	5%	10%	15%	20%
Rows:	1-4	5-8	1-4	5-8
10 Columns:	1. Spontaneous Release			
	2. Maximum Release			
	3. Melibiose			
	4. Lactose			

B. LDH Release Assay

15 1. General Procedures:

a) Prepare cells as for <sup>51</sup> CR Release assay, and labeled with LDH as per the manufacturer's instructions (Cytotox non-radioactive LDH release assay, Promega, USA)

20 b) To each well of a 96-well plate add (each reaction set up in quadruplicate):

25 - 25  $\mu$ l labeled cells  
- 5-20  $\mu$ l NHS  
- x  $\mu$ l sugar (final concentration of sugar:  
10<sup>-1</sup> to 10<sup>-3</sup> M)  
- RPMI/10% FCS (heat inactivated), to total volume of 100  $\mu$ l

30 c) Incubate plates at 37°C for 3 hr.  
d) Centrifuge plates at 1500 rpm for 5 min.  
e) Remove 50  $\mu$ l supernatant from each well (taking care not to remove any cells) and transfer to ELISA plate containing 50  $\mu$ l substrate mix (prepared according to manufacturer's instructions)  
35 f) Cover tray and incubate in the dark at room temperature for 30 min.

- g) Add 50  $\mu$ l stop solution to each well using multichannel pipette
- h) Read absorbance at 492 nm.

2. Controls:

- 5 a) Spontaneous release (no antibody or complement)
  - 25  $\mu$ l labeled cells
  - 75  $\mu$ l RPMI/10% FCS (heat inactivated)
- 10 b) Maximum release
  - 25  $\mu$ l labeled cells
  - 50  $\mu$ l 1% Triton X-100
  - 25  $\mu$ l RPMI/10% FCS (heat inactivated)

3. Calculation of % Lysis:

$$15 \quad \% \text{ Lysis} = \frac{\text{Experimental release cpm} - (\text{Spontaneous release cpm} + \text{sugar cpm})}{\text{Spontaneous release cpm}} \times 100$$

$$\text{Maximum release} - (\text{Spontaneous release cpm} + \text{sugar cpm})$$

4. Experimental Design:

Plate 1			
20	Columns: 1. spontaneous release	Rows: 1-4: cells + no sugar	
	2. maximum release	5-8: no cells + no sugar	
	3. 5% serum		
	4. 10% serum		
	5. 25% serum		
	6. RF10 alone		
25	Plate 2 melibiose		
	Plate 3 galactose		
	Plate 4 lactose		
	Plate 5 sucrose		
	Plates 6-9 same as plates 2-5 but no cells added		
30	Sugar Conc.		
	Columns: 1-2	1 $\times$ 10 <sup>-1</sup> M	Rows: 1-2 0% serum
	3-4	5 $\times$ 10 <sup>-2</sup> M	3-4 5% serum
	5-6	1 $\times$ 10 <sup>-2</sup> M	5-6 10% serum
	7-8	5 $\times$ 10 <sup>-3</sup> M	7-8 25% serum
35	9-10	2 $\times$ 10 <sup>-3</sup> M	
	11-12	1 $\times$ 10 <sup>-3</sup> M	

5. Preparation of Pig Spleen-Absorbed Rabbit Complement:

a) Cut pig spleen (obtained from local abattoir) into small pieces and prepare a single-cell suspension by passage through a fine metal sieve

5 b) Pellet cells by centrifugation at 700g, 7 min. at 4°C

c) Resuspend cell pellet in RPMI/10% FCS and repeat centrifugation

d) Resuspend in RPMI/10% FCS/10%

10 dimethylsulfoxide (DMSO)

e) Count cells and store frozen aliquots ( $3 \times 10^9$  cells/aliquot)

- use one aliquot for each absorption

f) For absorption, thaw and centrifuge at 600g, 5 min. at 4°C and remove the supernatant containing the DMSO

g) Wash two times with RPMI/10% FCS (10 ml)

h) Resuspend the cell pellet in rabbit complement; mix (rotary wheel) 2 hr. at 4°C

20 i) Centrifuge 600g, 5 min. at 4°C and remove the supernatant containing the rabbit complement; store at 4°C

II. RESULTS

Comparable results were obtained with both cell types (PAE's and PK<sub>1</sub>'s) using both lysis assays. The results of a typical lysis experiment are represented in Figure 2, in which the lysis of PAE's by human serum and by purified anti-GAL antibodies was determined using the <sup>51</sup>CR release assay. Comparable results were also obtained with PK<sub>1</sub> cells using the <sup>51</sup>CR release assay and with both cell lines using the LDH release assay. The results of these assays can be summarized as follows:

1. Xenoantibodies (NXAb) in human serum in the presence of complement are capable of lysing porcine

cells. Lysis increases with increasing concentrations of serum.

2. Pre-absorption of NHS with pig spleen cells (which removes the NXAB): No lysis.

5 3. Use of heat-inactivated complement: No lysis.

4. Use of NHS depleted of anti-GAL antibodies: No lysis.

5. Use of purified total anti-GAL antibodies 10 (IgG + IgM): Lysis.

6. Use of purified anti-GAL IgG: No lysis.

7. Use of purified total anti-GAL antibodies (IgG + IgM) and dithiothreitol (DTT): No lysis. (DTT is a reducing agent that disrupts the multimeric structure 15 of IgM antibodies without affecting IgG.)

Together these results demonstrate that the anti-GAL antibodies are responsible for the observed lysis. Purified anti-GAL IgG and DTT-treated total (IgG + IgM) anti-GAL antibodies failed to elicit lysis, indicating 20 that IgM, but not IgG, antibodies are causative agents in this system. Preliminary attempts to verify this observation using purified IgM prepared either in crude form by euglobulin fractionation or by  $\alpha$ -IgM affinity chromatography were unsuccessful. The inventors believe 25 this reflects inactivation of the IgM during preparation, rather than a true reflection of the capacity of anti-GAL IgM to cause lysis of porcine cells. heat inactivation of the complement prevented lysis, indicating that lysis of porcine cells is a complement-dependent phenomenon.

30 The effect of adding the disaccharide sugars melibiose (Gal  $\alpha 1 \rightarrow 6$  Gal) and lactose (Gal  $\beta 1 \rightarrow 4$  Glu) on the lysis of PAE's by human serum was assessed using the Cytotox non-radioactive LDH release assay. PAE's were incubated in the presence of 50% human serum as the

source of xenoantibody and complement, together with various concentrations of each sugar (1mM to 100mM). Under these conditions, melibiose, which has the Gal  $\alpha 1 \rightarrow 6$  Gal configuration, but not lactose, which has the 5 terminal Gal moiety in a  $\beta 1 \rightarrow 4$  configuration, protected the pig cells from lysis.

#### EXAMPLE 5

##### Inhibition of Human Serum-Induced Damage to Rat Hearts by Sugars

The Langendorf isolated perfused ex vivo heart 10 model was used to further demonstrate the involvement of anti-GAL xenoantibodies in hyperacute rejection.

#### I. METHODS

##### A. Preparation and storage of Human Plasma

1. Centrifuge fresh human blood at 3000 rpm, 10 min., 4°C to remove red blood cells (RBC's)
2. Remove the plasma
3. Centrifuge the plasma at 10,000 rpm, 10 min. 4°C to remove any remaining cells; decant the plasma
4. Add 2.5 ml of 0.1M EDTA pH 7.30 for 20 every 50 ml of plasma
5. Store 50 ml aliquots at -70°C
6. For heat-inactivated plasma, heat at 56°C for 60 min., then centrifuge at 2,500 rpm for 10 min.

##### B. Assessment of Complement Activity

Before being used in the ex vivo model, both heat inactivated and control plasma was tested for complement activity. Classical complement activity was determined by hemolysis using sensitized sheep RBC's as 30 described by Harrison and Lachman, In: Weir et al. (eds.), Handbook of Experimental Immunology and Immunochemistry, 4th Ed., Blackwell scientific Publications (1986). Alternative complement pathway

activity was determined using the rabbit hemolytic assay as described by Serrais et al., J. Immunol. Meth. 140: 93-100 (1991). The assay was performed in buffer containing EGTA and  $MgCl_2$ . The EGTA chelates the  $Ca^{++}$ , thus inhibiting the classical pathway. The  $Mg^{++}$  is required for activation and assembly of CdbBb, the alternative pathway C3 convertase.

5 C. Preparation of Plasma for Heart Perfusions

10 Plasma prepared from different blood packs is thawed at 37°C, pooled and filtered (100  $\mu m$  steel mesh, 8.0  $\mu m$  and 4.5  $\mu m$  Millipore filters, sequentially).  $CaCl_2$  is added at 0.58 mg/ml plasma, and the plasma kept on ice until ready for perfusion.

15 D. Ex Vivo Isolated Perfused Rodent Heart Model

1. Anesthetize rats with Nembutal (1  $\mu l$  sodium pentobarbitone (60 mg/ml)/g body weight) and mice with ether.

20 2. Surgically expose the heart and inject heparin (Porcine Mucous, 10,000 U/ml) into the femoral vein (rats: 0.3 ml injected).

3. Remove heart and place in ice-cold Krebs-Henseleit buffer containing heparin (0.2 ml/50 ml buffer).

Krebs-Henseleit buffer:

25 - 119 mM NaCl  
- 25 mM  $NaHCO_3$   
- 4.6 mM KCl  
- 1.2 mM  $MgSO_4 \cdot 7H_2O$   
- 1.3 mM  $CaCl_2 \cdot 2H_2O$   
- 1.2 mM  $KH_2PO_4$   
30 - 11 mM glucose  
- 0.25% (v/v) BSA  
- Adjust to pH 7.4; store at 4°C

4. Connect aorta to the canula of the Langendorff perfusion apparatus and tie firmly. The apparatus was 35 assembled by the present inventors according to experimental requirements of the Langendorff heart model as described in Doring & Dehnerrt, The Isolated Perfused

Heart According to Langendorf, Bionicsstechnik-Verlag  
March GmbH, D7806, West Germany.

5. Perfusion with Krebs-Henseleit buffer (made fresh each day), which is gassed continuously with 5 carbogen (95% O<sub>2</sub>, 5% CO<sub>2</sub>) at a pressure of 100 mmHg, at 37°C.

6. Attach a hook, connected to a transducer (Physiograph MK-111-S, Narco Bio-Systems) to the apex of the heart.

10 7. Perfusion heart for 20 min. with Krebs-Henseleit buffer to enable heart to stabilize (reservoir volume: 270 ml).

8. Add plasma (pre-warmed to 37°C) as follows:

15 - at 20 min. - add 10 ml plasma (= 5% plasma)  
- at 25 min. - add 10 ml plasma (= 9% plasma)  
- at 30 min. - add 10 ml plasma (= 13% plasma)

9. Monitor heart for a further 30 min. and record heart flow and contraction rate.

E. Sugar perfusion

20 1. Stabilize heart in Krebs Henseleit buffer for 30 min. as described above.

2. Add 2.5 ml of 1.08 M stock sugar solution to reservoir; total volume = 270 ml; final sugar concentration = 10mM.

25 3. Allow heart to restabilize for 10 min, then add plasma (control or heat inactivated) as per the schedule described above.

4. Record heart beat and flow rate.

F. Large-Scale Preparation of anti-GAL antibody-

30 Depleted Plasma

(all manipulations are performed at 4°C)

1. Start with 200 ml freshly prepared human plasma; 100 ml is subject to depletion; 100 ml is used as an untreated control from the same patient drawn on the same day; store at 4°C.

2. Filter the plasma sequentially through a 100  $\mu\text{m}$ , 8  $\mu\text{m}$  metal sieves and finally through a 0.45  $\mu\text{m}$  Millipore filter; dilute to 1000 ml with PBS, pH 8.0.

3. Concentrate to 200 ml using an Amicon 5 spiral wound cartridge (removes salt).

4. Equilibrate melibiose sepharose column (40 ml) with PBS, pH 8.0 (10 column volumes).

5. Passage the plasma through the melibiose sepharose column; collect the run-through and store at -10 70°C (=partially depleted plasma).

6. Wash column with PBS, pH 8.0 (10 column volumes) until the O.D. (280nm) of the eluate is approximately zero.

7. Combine the partially depleted plasma 15 and the eluate from the wash; concentrate to 200 ml (Amicon spiral concentrator).

8. Elute the anti-GAL antibody fraction with 4M guanidinium HCl pH 6.4 (2 column volumes).

9. Regenerate the column with PBS (10 20 column volumes).

10. Repeat the entire process an additional two times, i.e., repassage plasma through the melibiose column, wash, elute the anti-GAL antibody fraction and regenerate column.

25 11. For the anti-GAL antibody-depleted fraction:

- combine the eluate from the melibiose sepharose column with run-through from the final wash  
- adjust the volume to 5 liters with

30 Krebs Henseleit buffer and add EDTA to 10 mM; adjust pH to 7.0

- concentrate back to original volume (Amicon spiral concentrator); aliquot (35 ml) and store at -70°C

35 12. For the anti-Gal antibody fraction:

- combine the eluted anti-GAL antibody fractions, dilute to 5 liters with Krebs Henseleit buffer and add EDTA to 10 mM

5 - concentrate back to 10 ml (Amicon spiral concentrator); aliquot (1 ml) and store at -70°C

13. The anti-GAL antibody-depleted fraction and the purified anti-GA1 antibody fraction are tested for

a) Anti-GAL reactivity: Use as 10 primary reagents to stain porcine cells (PK<sub>1</sub>'s). Detect staining as described in Example 2, above. Analyze stained samples using a FACScan II (Becton Dickinson), according to the manufacturer's instructions.

b) Protein content: Determine using 15 the colorimetric method of Bradford, Anal. Biochem. 72: 248-54 (1976), with purified human IgG as the standard.

c) Electrolyte concentration: On the 20 day of the perfusion, the anti-GAL antibody depleted plasma is also tested to determine the calcium, magnesium and potassium levels using an electrolyte autoanalyser (Olympus); the levels of each are adjusted to normal as required.

## II. RESULTS

Rat hearts were connected to the Langendorff 25 apparatus and then stabilized by perfusion with Krebs Henseleit buffer for 10 min., and then a further 10 min. with the same buffer containing either melibiose or lactose (10mM). Human plasma was then added in stages as described above to a final concentration of 13 % and the 30 effect of the added sugar on cardiac function was assessed. The parameters measured were heart rate, amplitude (strength) of contraction and output (Figure 3).

In the presence of human serum alone (lower trace), the heart essentially stopped beating within minutes. The same result was obtained if lactose was added. In the presence of melibiose (upper trace) or 5 anti-GAL antibody-depleted plasma, however, the heart was able to maintain a strong beat. When the purified anti-GAL antibody was added back to the anti-GAL antibody-depleted plasma, the heart again stopped beating within minutes.

10

#### EXAMPLE 6

##### Characterization of the Porcine $\alpha$ -1,3-GalT Gene

cDNA's encoding porcine  $\alpha$ -1,3-GalT were generated by Polymerase Chain Reaction (PCR) technology. Total RNA of pig liver was isolated by homogenizing liver slices in 15 7M guanidinium thiocyanate, as described by Chomczynski & Sacchi, Anal. Biochem 162, 156-159 (1987); Sambrook et al., Molecular Cloning: A Laboratory Manual (2nd Edition), Cold Spring Harbor Laboratory Press (1989). Sixteen  $\mu$ g of the RNA, together with 1 $\mu$ g oligo dT primer, 20 were heat denatured for 5 minutes at 65°C prior to being transcribed into cDNA using avian myeloblastosis virus (AMV) reverse transcriptase in a 100 $\mu$ l reaction carried out at 37°C for 90 minutes. Three  $\mu$ l of the cDNA synthesis reaction was used in the subsequent PCR 25 amplifications. General procedures used for generation of cDNA are provided in Sambrook et al (1989), supra.

Primers for PCR were synthesized using phosphoramidite technology, on an Applied Biosystems DNA synthesizer. The sequence of the PCR primers was based 30 on identifying conserved regions within the published sequences for murine and bovine  $\alpha$ -1,3-GalT genes. Joziazze et al., J. Biol. Chem 264: 14290-97 (1989); Joziazze et al., Biol. Chem 267: 5534-5541 (1992). All primers were synthesized with EcoR1 linkers at the 5' end

for ease of cloning. In the following listing of the primers used in the present study, nucleotide positions varying between bovine and murine sequences are single-underlined; nucleotide positions varying between bovine 5 and human sequences are double-underlined:

Exon 2 primer (forward): 5'-

GTGAATTCAGCCCTGCCTCTTCTGCAG-3'

(SEQ ID NO: 1)

Designation: GTE2F -- 28-mer

10 - 1 difference b/w bovine & murine  
- no sequence available for human exon 2

Exon 4 primer (forward): 5'-

GTGAATTCAGGAGAAATAATGAATGTC-3'

(SEQ ID NO: 2)

15 Designation: GTE4F -- 28-mer

- no differences b/w bovine, murine & human

Exon 9 primer (reverse): 5'-

GTGAATTCGGGATCTGCCTGTACCC-3'

(SEQ ID NO: 3)

20 Designation: GTE9R -- 28-mer

- 3 differences b/w bovine & murine  
- 1 difference b/w bovine & human

3'-UTR primer (reverse): 5' GTGAATTCGAAATCATGGGAATTTACA-  
3'

25 (SEQ ID NO: 4)

Designation: GT3UR -- 28-mer

- no differences b/w bovine & murine  
- no differences b/w bovine & human

Exon 9 primer (forward): 5'-

30 AGGAATTCAGCATGATGCGCATGAAGAC-3'

(SEQ ID NO: 5)

Designation: GTE9F -- 28-mer

- no differences b/w bovine & murine
- 3 differences b/w bovine & human

PolyA primer (reverse): 5'-TTGAATTCTTTTTTTTV\*N\*\*-3'  
(SEQ ID NO: 6) \* V = A or C or G; \*\* N = A or C or G  
5 or T (primer includes all nucleotide variants for V and N)

Designation: APATR -- 23-mer

The PCR conditions used to generate porcine  $\alpha$ -1,3-GaLT cDNA fragments were as

10 follows:

- 1) For GTE2F + GTE9R and GTE4F + GTE9R: heat to 94°C (60 seconds); then proceed with 35 reiterations (cycles) of the following three steps: (1) 94°C, 40 seconds, (2) 57°C, 50 seconds, and (3) 72°C, 80 seconds.
- 15 2) For GTE9F + GT3UR: heat to 94°C (120 seconds); then proceed with 35 cycles of: (1) 94°C, 40 seconds, (2) 48°C, 45 seconds, and (3) 72°C, 60 seconds.

The PCR fragments were subcloned into EcoR1-restricted pBluescript II KS+ (Stratagene, Cat, # 2 20 12206) and the DNA sequence was determined using the chain termination method. The DNA sequence was assembled and analyzed using DNASIS-Mac v2.01 (Hitachi)

The nucleotide sequence of porcine  $\alpha$ -1,3-GaLT (SEQ ID NO: 7) and the derived amino acid sequence (SEQ ID NO: 10) of the enzyme are shown in Figures 4 and 5. A single large open reading frame extends from the initiating methionine at nucleotide 91 to a stop codon located at nucleotide 1204. The sequence surrounding the putative initiating methionine conforms to the consensus 30 eukaryotic initiation sequence. Kozak, Cell 44, 283-92 (1986).

The porcine cDNA sequence is compared to the corresponding murine (SEQ ID NO: 9) and bovine (SEQ ID NO: 8) sequences in Figure 4. The locations of introns 35 within the murine gene are also shown. Joziazze et al., J. Biol. Chem 267: 5534 (1992). This alignment

demonstrates that exon 3, located within the 5' untranslated region of the mouse gene, is not found in either the porcine or bovine cDNAs. The overall sequence identities between the coding sequences are as follows:

5 a) pig compared to mouse:- 75.02% (exon 3 not considered)

b) pig compared to bovine:- 85.15%

The amino acid sequences of the porcine (SEQ ID NO: 10), murine (SEQ ID NO: 12) and bovine (SEQ ID NO: 11)  $\alpha$ -1,3-Galt enzymes are depicted in Figure 5. The locations of introns are also shown, based on their positions within the mouse gene (Joziasse et al., 1992). This alignment illustrates that the overall amino acid homologies are:

15 a) pig compared to mouse: 71.98%

b) pig compared to bovine: 82.87%

c) bovine compared to mouse: 73.72%

#### EXAMPLE 7

##### Identification of Potential Sites to Interrupt the $\alpha$ -1-3-Galt Gene

20 The present inventors' choice of a site for interrupting the  $\alpha$ -1,3-Galt gene has been influenced by several characteristics of the gene and its expression. In particular, several mRNAs for  $\alpha$ -1,3-Galt have been detected in the mouse. Joziazze et al., J. Biol. Chem. 25 267: 5534 (1992). These mRNAs are products of alternative splicing events in which exons 5 and/or 6 may be deleted. Hence, these exons are not appropriate interruption sites in the mouse, since a transcript encoding a functional  $\alpha$ -1,3-Galt enzyme presumably could 30 be formed when exons 5 or 6 are spliced out. Moreover, the present inventors have isolated two different classes of  $\alpha$ -1,3-Galt cDNA clones from the pig - one that includes exon 5 and one with exon 5 deleted. It is possible that mRNA's with and without exon 6 are also

formed by alternative splicing in the pig. Thus, for initial experiments the present inventors have not chosen these exons as sites for interruption.

Insertion of an interrupting-DNA fragment into 5 exon 4 (which encodes the cytoplasmic NH<sub>2</sub>-terminal domain and the membrane-anchoring domain; see Figure 5) would disturb production of a transcript encoding an active  $\alpha$ -1,3-GaIT. Hence this exon is an appropriate site to disrupt the  $\alpha$ -1,3-GaIT gene. Similarly, exons 7 and 8, 10 which encode the NH<sub>2</sub>-terminal region of the catalytic domain, are suitable disruption sites. Insertion of a interrupting DNA fragment within these exons would prevent the synthesis of an active catalytic domain.

A preferred site for interrupting the mouse gene 15 is located at a SalI site found within exon 9 of the mouse  $\alpha$ -1,3-GaIT gene, at codons 221 + 222 (see Figure 5). This site is positioned 150 amino acids from the COOH-terminus, within the catalytic domain. The mouse gene within the present inventors' constructs for 20 homologous recombination is interrupted at this SalI site. The amino acids encoded by nucleotides at this SalI site are conserved in the pig and bovine sequences, although the SalI site itself is not. Construction of a SalI site at this position in the pig gene (e.g., by *in* 25 *vitro* mutagenesis) provides a useful construct to inactivate the gene.

#### EXAMPLE 8

##### Choice of a DNA Fragment to Interrupt the $\alpha$ -1,3-GaIT Gene

The present inventors have used both the neomycin 30 resistance (neo<sup>R</sup>) gene and the hygromycin resistance gene (hyg<sup>R</sup>) to interrupt the  $\alpha$ -1,3-GaIT gene. In one set of "knockout" constructs the neo<sup>R</sup> and hyg<sup>R</sup> genes are linked to the murine phosphoglycerate kinase (PGK) promoter (Adra et al., Gene 60: 65-74 (1987) and are both bordered

by polylinker sequences that include restriction sites for EcoRV and ClaI.

In another construct, expression of the neo<sup>R</sup> gene is directed by an altered polyoma virus promoter (PMC1; Thomas and Cappelli, cell 51: 503-12 (1987)). In this construct the present inventors have addressed the problem of including an antibiotic resistance gene within the genome of transplant organs. That is, in some circumstances it may not be desirable to have genes conferring resistance to antibiotics present in the organ to be transplanted. The FLP/FRT recombinase system of yeast has been used to eliminate the neo<sup>R</sup> gene from the sequence that interrupts the  $\alpha$ -1,3-GalT gene.

In a construct of the present invention, the neo<sup>R</sup> gene is bordered at both the 5' and 3' ends by FRT DNA elements. In addition, stop codons for each of three reading frames have been inserted 3' to the neo<sup>R</sup> gene, and these stop codons, together with a single FRT sequence, will remain within the  $\alpha$ -1,3-GalT gene after the neo<sup>R</sup> gene has been excised by FLP. Targeted cells carrying a genomic copy of the neo gene flanked by direct repeats of the FRT could be supplied with FLP recombinase in two ways:

1) Introduction into cells of partially purified FLP protein:

FLP protein (0.1 - 10  $\mu$ g) is introduced ("transfected") into approximately  $10^7$  cells using standard electroporation conditions. The cells are plated out into gelatinized tissue culture dishes in appropriate medium, at a sufficient dilution to result in individual colonies. Approximately 200 of these colonies are then picked for further analysis.

2) Transfection with plasmids containing the FLP gene:

A plasmid containing the FLP gene under control of a promoter able to drive FLP expression, e.g., the human interferon-inducible 6-16 promoter, is constructed according to standard methods. Porter et al., EMBO J. 7: 5 85 (1988). Approximately 10  $\mu$ g of FLP expression plasmid is transfected into approximately  $10^7$  cells using standard electroporation conditions. With a plasmid containing the human 6-16 promoter, interferon is added at approximately 500 units/ml, in order to induce 10 expression of FLP. The cells are then treated as in (1), above.

The procedure to knock out the  $\alpha$ -1,3-GaIT gene in ES cells using an FRT-containing construct is:

- a) electroporate the complete construct into ES 15 cells
- b) select neo<sup>R</sup> cells, and identify those ES cells having an interrupted  $\alpha$ -1,3-GaIT gene
- c) delete the neo<sup>R</sup> gene using FLP recombinase, as described above; cells are tested for the excision 20 event as follows:

First, samples of each selected cell line are tested for the absence of the neo<sup>R</sup> gene by treatment with the chemical G418. The cells will die in the presence of approximately 200  $\mu$ g/ml G418 unless the neo<sup>R</sup> gene is still 25 present in the genome. Cell lines that are G418 sensitive are then tested further to confirm that excision of neo<sup>R</sup> has occurred. This is done by Southern analysis or PCR analysis, both described in Sambrook et al. (1989). For Southern analysis, genomic DNA is 30 isolated from the cells, digested with an appropriate restriction enzyme, subjected to agarose gel electrophoresis, and the digested DNA transferred to a membrane. The DNA is hybridized with a labeled probe, the label is detected (e.g., with X-ray film or color 35 development), and the pattern of bands indicates whether

or not an excision event had occurred in the cell line. For PCR analysis, genomic DNA is isolated from the cells and subjected to PCR reaction with suitable oligonucleotide primers.

5 d) following confirmation of neo<sup>R</sup> excision, the manipulated ES cells or PGC's are used to generate chimeric animals.

EXAMPLE 9

Preparation of DNA Constructs to Interrupt the  $\alpha$ -1,3-GaIT  
10 Gene in Mice

Gene targeting (homologous recombination) is more efficient if the cloned cDNA fragments used for targeting are isolated from the cell line which is used for the gene knockout (i.e., the DNA is "isogenic").

15 Accordingly, DNA was isolated from the E14 ES cell line (Hooper et al., Nature 326: 292-95 (1987)) and used to construct a mouse genomic library. The DNA was digested partially with the restriction enzyme Sau 3A, and fragments 12 kb - 20 kb in size were isolated by glycerol

20 gradient fractionation. The size-fractionated DNA was ligated into the Bam H1 site of  $\lambda$ EMBL3 (Sambrook et al. 1989, supra), and packaged *in vitro* to form lambda phage particles. The lambda library was plated by infection of *E. coli* strain PMC103 host cells (Doherty et al., Gene 25 124: 29-35 (1993)) at a density of  $4 \times 10^4$  phage per plate. A bovine cDNA clone, about 900 bp in length and containing a portion of the  $\alpha$ -1,3-GaIT gene corresponding to exons 7 - 9, was used to probe a total of  $5.6 \times 10^5$  independent recombinant phage. Four overlapping clones

30 containing  $\alpha$ -1,3-GaIT gene sequences were isolated and purified. The SalI restriction sites within these clones were mapped (Figure 6), and the 4.0kb, 5.5kb, 11kb and 12kb SalI fragments from two of the clones ( $\lambda$ 3 and  $\lambda$ 5) were subcloned into pBlueScript KS+ (Stratagene) or pUBS

(pUC19 carrying the pBlueScript KS+ polylinker) to facilitate further detailed mapping of restriction sites.

These four subclones (designated p $\alpha$ GT-S4.0, p $\alpha$ GT-S5.5, p $\alpha$ GT-S11 and p $\alpha$ GT-S13) were mapped for restriction sites with restriction enzymes BamHI, EcoRI, HindIII, XbaI, XhoI, KpnI, SacI, SacII, EcoRV, PstI, SmaI, NotI and BglII. p $\alpha$ GT-S4.0 and p $\alpha$ GT-S5.5 were also checked for PvuI, PvuII, NdeI and SphI restriction sites. Detailed restriction maps of the 4 subclones were drawn from these data (Figures 7-12).

On the basis of these maps a knockout strategy was conceived. Basically the strategy is to insert a resistance gene (either neo<sup>R</sup> or hyg<sup>R</sup>) into the SalI site which lies within Exon 9. The knockout construct carries the 4.0 and 5.5kb SalI fragments from p $\alpha$ GT-S4.0 and p $\alpha$ GT-S5.5 which flank the Exon 9 SalI site (Figure 13). Screening for homologous recombination events then can be carried out using a DNA fragment representing the genomic region but lying outside the DNA included in the knockout construct, i.e., outside the 9.5kb covered by p $\alpha$ GT-S4.0 and p $\alpha$ GT-S5.5. A 0.7kb EcoR1/XmnI fragment from p $\alpha$ GT-S11 is used to screen Southern blots of BglII digested ES cell DNA for homologous recombinant events. An 8.3kb band should appear on these Southern blots when the uninterrupted  $\alpha$ 1,3-GalT gene is probed with this EcoR1/XmnI fragment (Figure 14). Insertion of the neo<sup>R</sup> gene after a homologous recombination event will give rise to a 6.4kb band, due to the presence of a BglII site just flanking the Exon 9 SalI site within the knockout construct. Thus the presence of the 6.4kb band is diagnostic for a homologous recombination event.

To carry out this strategy, the present inventors prepared a series of knockout constructs. The generation of one such construct is outlined in detail in Figure 15. The vector p $\alpha$ GT-S5.5, which carries the 5.5kb fragment

immediately 3' to the Exon 9 SalI site, was chosen as the starting vector. p $\alpha$ GT-S5.5 was digested with EcoRV and ClaI, generating a vector with a blunt end and a ClaI compatible end. A 1.3kb fragment carrying the PMC1 5 promoter-driven neo<sup>R</sup> gene flanked by FRT sites was excised from plasmid pNeo2FRT (previously constructed by the present inventors) by digesting with BamHI, filling in the restriction site and then digesting with ClaI to generate a fragment with one blunt end and one ClaI 10 compatible end. The nucleotide sequence of this 1.3kb fragment is provided in Figure 16 (SEQ ID NO: 13). This fragment was then ligated into the ClaI/EcoRV digested p $\alpha$ GT-S5.5, the ligation mix transformed and colonies screened for recombinants. One colony was recovered that 15 contained the Neo<sup>R</sup> fragment inserted into the EcoRV/ClaI of p $\alpha$ GT-S5.5, based on the restriction pattern after digestion with diagnostic restriction enzymes ClaI, EcoRV, XbaI and EcoRI. This construct was designated PNeo $\alpha$ GT6.8.

20 pNeo $\alpha$ GT6.8 was digested with SmaI, generating a vector with blunt ends. The 4.0kb SalI fragment was excised from p $\alpha$ GT-S4.0 and the ends filled. This fragment was then ligated into the SmaI digested p $\alpha$ GT-S5.5, the ligation mix transformed and colonies screened 25 for recombinants. Four colonies were recovered which contained the 4.0kb SalI fragment inserted into the SmaI sites of pNeo $\alpha$ GT6.8 with the 5' portion of Exon 9 lying near the 3' portion of the exon in the nearby SalI 5.5kb fragment. The identity and orientation of the insert was 30 confirmed by the restriction pattern after digestion with diagnostic restriction enzymes XbaI, EcoRI, HindIII, BamHI, EcoRV and others. This construct was designated pNeo $\alpha$ GT10.8.

35 pNeo $\alpha$ GT10.8 was digested with ClaI, generating a vector with ClaI compatible ends. Two complementary

oligomers were synthesized that, when annealed, generated a linker containing translation termination codons in all three reading frames and a BglII site. The linker has ClaI compatible ends. The linker was ligated into the 5 ClaI digested pNeo $\alpha$ GT10.8, the ligation mix transformed and colonies screened for recombinants. Many colonies were recovered that contained the linker inserted into the ClaI sites within pNeo $\alpha$ GT10.8 based on the restriction pattern after digestion with diagnostic 10 restriction enzymes Bg1III, Cla and Bg1III/NotI. This construct has been sequenced to confirm the identity, copy number and orientation of the insert. This construct is called pNeo $\alpha$ GT10.8B (Figure 17).

EXAMPLE 10

15        ES Cells - General Materials and Methods  
              Working Conditions

Procedures for the isolation and culturing of all cell lines (embryonic stem, primordial germ and fetal fibroblast cell lines) require aseptic conditions to 20 prevent growth of contaminating organisms:

1. All laboratory bench tops and equipment are wiped down with 70% ethanol prior to use.
2. All surgical instruments are autoclaved prior to use.
- 25 3. Water for media preparation and cleaning of glassware is of high quality (e.g., Milli-Q water, prepared by passage through a Milli-Q ultrapure water system (Millipore)).
4. Glassware is either dry-heat sterilized or 30 autoclaved following extensive cleaning in Milli-Q water before use.
5. All tissue culture work is carried out under laminar flow conditions (Hepa filtered horizontal laminar flow workstation).

6. All media are filter sterilized (22 $\mu$ m disposable filter) prior to use.

7. Antibiotics are used to minimize the risk of bacterial contamination (Penicillin, Streptomycin and

5 Gentamicin for bacteria; Nystatin for fungi).

#### Media/Solution Preparation

##### DULBECCOS MODIFIED EAGLE MEDIUM (DMEM)

10.0g DMEM powder- Gibco (the low-glucose or  
high-glucose formulation, with or without  
10 pyruvate, may be used; L-glutamine is  
included)  
1.0 liter Milli-Q-Water  
3.7g NaHCO<sub>3</sub>  
Stir slowly until dissolved  
15 Adjust pH ~ 7.2  
Filter sterilize (following filter sterilization pH to  
rises to 7.4)  
Keep at 4°C.

##### STO CELL MEDIUM

20 83.0 ml DMEM  
15.0 ml 15% fetal bovine serum (FBS); batch tested  
before use  
1.0 ml Pen/Strep 1:100  
1.0 ml Glutamine 1:100 (if needed) (see note below)  
25 Filter sterilize and keep at 4°C.

Note: Replenish complete medium (DMEM medium) (STO or  
ES) with glutamine.

\*This step is only required if medium is older than 1  
week - 10 days, as the glutamine breaks down after this

30 time.

##### ES CELL MEDIUM WITH OR WITHOUT LIF

up to 100.0 ml DMEM  
15.0 ml 15% FBS (batch tested before use;  
see below)  
35 1.0 ml (from 0.01M stock)  $\beta$ -mercaptoethanol (0.1 mM  
final concentration)  
1.0 ml Pen Strep. 1:100  
0 - 1.0 ml Glutamine 1:100 (if needed)  
1.0 ml Nystatin 1:100

0 - 2.5 ml Recombinant murine LIF (from  $4 \times 10^4$   
U/ml; 1000U/ml stock); activity-tested  
using LIF Assay (see below)

0.4 ml Gentamicin

5 1.0 ml Nucleotides

1.0 ml Non-essential amino acids

PENICILLIN/STREPTOMYCIN ANTIBIOTIC SOLUTION (1:100)  
- Commonwealth Serum Laboratories, Australia;  
Catalogue No. 05081901

10 Penicillin G - 5000 U/ml  
Streptomycin Sulphate - 5000  $\mu$ g/ml.

#### MITOMYCIN-C SOLUTION

2.0 mg Mitomycin-C (Sigma Chemical Co. ("Sigma");  
Catalogue No. M0503)

15 200.0 ml STO Cell Medium

Filter sterilize, divide into 20x 10 ml aliquot's and  
store at -20°C.

#### PHOSPHATE BUFFERED SALINE (PBS)

For 100 ml Milli-Q Water: (Ca<sup>++</sup> and Mg<sup>++</sup> - containing)  
20 (Ca<sup>++</sup> and Mg<sup>++</sup> - free)

NaCl	0.89	0.80
KCl	0.02	0.02
KH <sub>2</sub> PO <sub>4</sub>	0.02	0.02
Na <sub>2</sub> HPO <sub>4</sub> · 12H <sub>2</sub> O	0.289	1.115
25 CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.014	-
MgCl <sub>2</sub> · 6H <sub>2</sub> O	0.01	-
Na pyruvate	0.0036	-
D-glucose	0.1 g	-

Adjust to pH 7.4 and filter sterilize

30 (Ca<sup>++</sup> and Mg<sup>++</sup> - free PBS is purchased from ICN Cell  
Biology and Tissue Culture, Cat. No. 18-604-54)

#### TRYPSIN/VERSENE (TV) WORKING SOLUTION (TV x 1)

In PBS (Ca<sup>++</sup> and Mg<sup>++</sup> - free):  
35 0.25% (w/v) trypsin (lyophilized)  
0.04% (w/v) EDTA or EGTA

or:

To 1 liter of milli-Q water add the following:

	Trypsin powder (Porcine, Difco)	2.5 g
	EDTA or EGTA	0.4 g
	NaCl	7.0 g
5	Na <sub>2</sub> HPO <sub>4</sub> 12H <sub>2</sub> O	0.3 g
	KH <sub>2</sub> PO <sub>4</sub>	0.24 g
	KCl	0.37 g
	D-Glucose	1.0 g
	Tris	3.0 g
10	Phenol red	1.0 ml

Adjust to pH 7.6, filter sterilize, aliquot and store frozen.

EGTA: Ethylene-glycol-bis(β-amino-ethyl ether)N,N,N',N"-tetra-acetic acid [Ethylene-bis(oxy-15 ethylenenitrilo)]tetraacetic acid

EDTA: Ethylenediaminetetraacetic Acid

Use either EDTA or EGTA. EGTA is preferred as it is less damaging to the ES/PGC cells.

#### GELATIN WORKING SOLUTION

20 0.1% gelatin in Milli-Q Water

Dissolve gelatin by heating to 60°C.  
Filter sterilize when still warm.

To gelatinize tissue culture plates:

1. Cover dish with solution, leave 30 minutes

25 2. Aspirate gelatin and let dish air-dry.

#### NUCLEOSIDE STOCK SOLUTION

	Milli-Q Water	100 ml
	Adenosine (Sigma)	80 mg
	Guanosine (Sigma)	85 mg
30	Cytidine (Sigma)	73 mg
	Uridine (Sigma)	73 mg
	Thymidine (Sigma)	24 mg

1. Dissolve by warming to 37°C.

2. Filter sterilize and aliquot while warm.

35 3. Store at 4°C or -20°C.

4. Thawing of nucleotides for use in ES cell media  
(a) nucleotides come out of solution upon  
thawing;  
(b) Warm to 37°C to resolubilize before use.

5 NON-ESSENTIAL AMINO ACIDS (1:100)

- Commonwealth Serum Laboratories; Catalogue No. 09751301

100x concentrate for minimum essential medium (Eagle):  
(1.0 ml is added to 100 ml ES Cell Medium)

mg/10 ml milli-Q H<sub>2</sub>O

10	Glycine	7.5
	L-Alanine	8.9
	L-Asparagine · H <sub>2</sub> O	15.0
	L-Aspartic Acid	13.3
	L-Glutamic Acid	14.7
15	L-Proline	11.5
	L-Serine	10.5

WHITTEN'S CULTURE MEDIUM

	KCl	0.0356
	KH <sub>2</sub> PO <sub>4</sub>	0.0162
20	MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.0294
	NaCl	0.4
	NaHCO <sub>3</sub>	0.2106
	Glucose	0.1
	Na Pyruvate	0.0036
25	Ca Lactate 5H <sub>2</sub> O	0.0527
	Na Lactate	0.2416 ml
	Milli-Q-H <sub>2</sub> O	100 ml

The solution is adjusted to a final milliosmolarity of 250-280 by addition of H<sub>2</sub>O or NaCl.

30 Filter sterilize and store at 4°C for two weeks.

Working solution:

10 ml Whitten's medium  
1.5g BSA fraction V (Miles Pentex,  
Diagnostic division,  
Kankakee, Ill., USA;  
35 Code No. 81-001-4)

Filter Sterilize and equilibrate in 5%O<sub>2</sub>:5%CO<sub>2</sub>:90% N<sub>2</sub> at 39.5°C, 95% humidity.

FBS BATCH TRIALS

Batches of FBS vary in the ability to support growth of ES cells, and in the ability to maintain the undifferentiated state of such cells. The following 5 procedure is used to identify suitable batches of FBS. Use ES cells from between 2 & 20 passages:

Day 1

10 Split ES colonies and plate into dishes without feeder cells but with LIF. Incubate for 3 days.

Day 4 Trypsinise to detach colonies and cells. Count cells and dispense into gelatinized 6cm dishes containing ES Cell Medium and LIF (no serum added) as follows:

	Dish Number	No. Cells	Batch FBS	Control Serum (Batch Tested)
	Non-Inactivated Serum		A	B
20	1	250	5 ml	-
	3	250	-	5 ml
	5	250	-	-
	7	2000	5 ml	-
	9	2000	-	5 ml
25	11	2000	-	-
	Inactivated Serum, as control (56°C for 15 min.)			
	13	250	5 ml	-
	15	250	-	5 ml
30	17	250	-	-
	19	2000	5 ml	-
	21	2000	-	5 ml
	23	2000	-	-

There are duplicate plates for each treatment.

35 Incubate low density dishes for 5 days  
Incubate high density dishes for 3 days

Day 7 Fix high density cells and stain with hematoxylin.

Day 9 Fix low density cells and stain for alkaline phosphatase.

#### LIF ASSAY

This procedure is used to assay the potency of Leukaemic Inhibitory Factor (LIF).

5 Day 1 Split one 10 cm dish of confluent STO cells into five dishes. Incubate for 2 - 3 days in STO medium.

Day 3/4 When cells are confluent, replace medium with DMEM + 10% FBS. Incubate for 3 days.

10 Day 6/7 Collect conditioned medium (CM) and store at 4°C.

\*Prepare low density ES cell cultures as described above.

	Dish	No. Cells	C.M.	Medium	1000 U/ml	Medium w/o LIF	Presumed LIF Content
15	1,2,3	250	0.1 ml	4.9 ml	-	-	200 U/ml
	4,5,6	250	0.25 ml	4.75 ml	-	-	500 U/ml
	7,8,9	250	0.5 ml	4.5 ml	-	-	1000 U/ml
	10,11,12	250	1.0 ml	4.0 ml	-	-	2000 U/ml
	13,14,15	250	-	-	5 ml	-	
20	16,17,18	250	-	-	-	5 ml	

There are triplicate plates for each treatment.

Fix and stain for alkaline phosphatase.

#### Preparation of Fibroblast Feeder Cell Layers

25 Embryonic pluripotential cells are cultured in vitro on a layer of fetal fibroblast cells. The fibroblast cells provide a wide range of factors necessary for the growth of pluripotential embryonic cells (e.g. growth factors, cytokines, factors that are essential for maintenance of ES cell pluripotency).

#### 30 ISOLATION OF PORCINE FETAL FIBROBLASTS:

1. Remove developing porcine fetuses (preferably between days 16-30 of development) from uterus by aseptic dissection.
2. Remove skin layer from fetus.

3. Dissect out soft tissue avoiding developing viscera. The white (fibroblast containing) tissue is found just under the skin layer.
4. Wash dissected tissue in PBS (Ca<sup>++</sup> and Mg<sup>++</sup> free).
5. Centrifuge at 1000 rpm for 5 min.
5. Remove supernatant.
6. Incubate tissue in Trypsin/Versene Working Solution for 20 min.
7. Dissociate cells by vigorously pipetting.
10. Centrifuge at 1000 rpm for 5 min.
8. Remove supernatant.
9. Resuspend cells in STO Cell Medium. Allow large cell-clumps to settle.
10. Plate out cells within supernatant (i.e., large cell clumps are not included) onto gelatinized tissue culture plates. Incubate cells in an atmosphere of 5% CO<sub>2</sub>, 95% air (37.5°C, 95% humidity) until a confluent layer of fibroblast cells appears (~4-5 days).
- 20 11. Passage of cells may be continued to increase cell numbers, or cells may be frozen or inactivated for further use.

CULTURE OF FETAL FIBROBLAST FEEDER LAYERS FROM FROZEN STOCKS:

25 Several different types of mouse feeder (STO cells) and porcine and bovine fetal fibroblasts can be used to form feeder layers. These include:

- (1) Bradley/Baylor mouse STO feeder cells that have been modified to express human LIF (gift from Allan Bradley, Institute for Molecular Genetics, Baylor College of Medicine, Texas Medical Center, Houston, Texas, USA)
- (2) Robertson/Columbia mouse STO feeder cells that have been modified to express murine LIF (gift from Elizabeth Robertson, Columbia University, New York, USA)
- (3) Several porcine fetal fibroblast lines
- (4) Several bovine fetal fibroblast lines

(the fibroblast lines of (3) and (4) were derived by the present inventors using the procedures described above)

The procedure for producing feeder layers is as follows:

- 5 1. Rinse one 10 cm tissue culture (tissue cure) dish with gelatin/Milli-Q water solution for 30 min. Aspirate gelatin solution and let dish air-dry.
3. Add 10 ml of STO cell medium to 15 ml centrifuge tube.
- 10 4. Remove feeder layer cells frozen in freezing media from liquid N<sub>2</sub> container.
5. Thaw cells by warming vial in hands or in 37°C water bath.
6. Transfer STO cells to medium in centrifuge tube.
- 15 7. Spin at 1000 rpm for 5 min.
8. Resuspend cells in 10 ml medium and transfer to gelatin-treated tissue culture dish.
9. Incubate at 37°C for 3 days.

SPLITTING OF FEEDER LAYER STO CELL/FETAL FIBROBLASTS:

- 20 This procedure is used to expand the number of cells from a single confluent plate/dish; cells are detached from the confluent plate and transferred to fresh plates at sub-confluent densities.
  1. Gelatinize five 10 cm tissue culture dishes.
  - 25 2. Examine incubated STO cells under microscope and check for confluence.
  3. If STO feeder monolayer is confluent (cells cover bottom of dish, or nearly so), wash gently with PBS (Ca<sup>++</sup> and Mg<sup>++</sup> - free) for 1 min.
  - 30 4. Aspirate PBS and add 1 ml Trypsin/Versene Working Solution for 1 min (or until cells start to detach). Check under microscope.
  5. Detach cells by vigorously pipetting, add 1.0 ml STO Cell medium (i.e., a ratio of 1:1 STO Cell

medium:Trypsin/Versene Working Solution) to neutralize trypsin, and transfer to a centrifuge tube containing 10-15 ml STO Cell medium. Wash cells remaining on dish with some of STO cell medium from the tube. Centrifuge at 1000 rpm for 5 min., aspirate supernatant, resuspend pellet in 1 ml STO Cell medium. Resuspend cells to make single cell suspension. Make up to 50 ml with STO Cell medium.

5. Dispense 10 ml into each of the five tissue culture dishes and incubate until confluent (~ 3 days).

10

**INACTIVATION OF FEEDER LAYERS:**

The present inventors use two alternative methods for inactivating feeder layers, which stops the cells from dividing:

15 (1) Mitomycin treatment:

1. Check dishes for confluence of STO cells/fetal fibroblasts.
2. Thaw mitomycin-C solution and use undiluted.
3. Aspirate STO cell medium from feeder cell plate.
- 20 4. Add 10 ml aliquot of mitomycin-C to plate and incubate at 37°C for 1-3 hours.
5. Aspirate mitomycin-C, wash cells in 1x PBS (without  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$ ) for 1 min.
6. Aspirate PBS and add 1 ml trypsin solution for 1

25 min.

7. Detach cells by vigorously pipetting and transfer to STO cell medium in centrifuge tube.
8. Centrifuge at 1000rpm for 5 min.
9. Resuspend cell pellet in 1 ml ES Cell Medium.
- 30 10. Plate out in dishes in preparation for addition of ES cells.

(2) Gamma Irradiation:

1. Check dishes for confluence of STO cells/fetal fibroblast.

2. Trypsinise cells into single cell suspension.
3. Irradiate cells (3000 rads) in STO cell medium.
4. Centrifuge at 1000 rpm for 5 min.
5. Resuspend pellet in 1 ml ES Cell Medium.
6. Transfer cells to gelatinized tissue culture dishes with ES Cell Medium and place in incubator at 37°C until the cells adhere to the dish. NOTE: If cells are not confluent, count using hemocytometer and seed at  $5 \times 10^4$  cells in 1 ml medium per well of Nunc 4-well plate.
- 10 One 10 cm dish of inactivated cells can be split into:  
Ten 4-well plates (Nunc tissue culture plates), or Eight 3.5 cm tissue culture dishes, or
- 15 Three 6 cm tissue culture dishes, or Two 20 cm tissue culture dishes.

Demonstration of Totipotency

A. Blastocyst Injection

The ability of embryonic cell lines to form germline 20 chimeric animals is a conclusive test for their totipotency. This can be accomplished by blastocyst injection experiments, using techniques for various mammalian species substantially the same as those established for the mouse. See Example 14, below. See 25 also, e.g., Bradley, Production and Analysis of Chimeric Mice, In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach (E.J. Robertson, ed.), IRL Press, Oxford, pp. 113-52 (1987). However, for porcine manipulations the holding pipette must be somewhat larger 30 as porcine embryos are larger than mouse embryos.

B. Co-Culture of ES Cells/PGC's and Morula Embryos

Embryos at the morula stage of development are surgically collected from superovulated animals. For

porcine embryos, for example, the zona pellucida is then disrupted using Acid Tyrodes solution and ES cells/PGC's are cultured in the presence of the zona pellucida-disrupted morulae. ES/PGC cells adhere to the exposed 5 morula cells and, following overnight culture in Whitten's medium, the embryos are transferred to synchronized recipients. Preferably, the zona pellucida-disrupted morula is completely free of the zona pellucida. However, this need not be the case as long as 10 the ES cells/PGC's can gain direct access to at least some of the morula cells.

C. Morula Injection

ES cells and PGC's can be injected into a morula embryo prior to formation of the blastocyst cavity. The 15 technique is similar to blastocyst injection. ES cells or PGC's are drawn into an injection pipette, which is inserted beneath the zona pellucida. Then, the cells are expelled so that they are in contact with the cells of the morula embryo. The injected morula is then cultured 20 overnight in Whitten's medium (porcine) or other appropriate medium to allow blastocyst formation.

D. Nuclear Transfer and Embryo Cloning

ES cells and PGC's can be fused to enucleated zygotes that have been derived by in vitro maturation, in 25 vitro culture, in vitro fertilization or collected surgically. Following successful fusion the embryos can be transferred to synchronized recipients. In vitro or in vivo-collected porcine oocytes, for example, are manipulated in Whitten's medium supplemented with 1.5% 30 BSA Fraction V and 7  $\mu$ g/ml cytochalasin B (Sigma). A bevelled micropipette is used to remove the metaphase plate from the oocyte. A single ES cell or PGC (after trypsin treatment to form a single-cell suspension) is

inserted through the zona using a bevelled micropipette, such that the cell comes in contact with the oocyte plasma membrane. Fusion is achieved in a 28 V/cm AC field for 5 sec. followed by an 80 V/cm DC pulse of 100 5  $\mu$ sec. duration. Subsequent to observed fusion, embryos are incubated at 39° C in 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub> in microdrops of Whitten's medium supplemented with 1.5% BSA, until transfer to a synchronized recipient.

EXAMPLE 11

10 Murine ES Cell Culture

ES cells are able to differentiate spontaneously into many different cell types, and culture conditions which prevent this differentiation are critical for the continuous passage of these cells in an undifferentiated. 15 form, capable of contribution to chimeric mice.

I. CULTURE CONDITIONS

ES cells are grown in polystyrene cell culture dishes treated with 0.1% gelatin (made up in PBS or Milli-Q water) for 10 minutes. A feeder layer of 20 mitotically inactivated fibroblasts provides a source of cytokines. The fibroblasts are either primary mouse embryo fibroblasts (PMEFs), or STO fibroblasts, an immortal line. The medium used is DMEM supplemented with glucose, amino acids and nucleosides. Robertson, Embryo-

25 Derived Stem Cell Lines. In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach (E.J. Robertson, ed.), IRL Press, Oxford (1987). To this medium is added LIF (final concentration of 10<sup>3</sup>U/ml Esgro, AMRAD). FBS is added to 15%. The batch of FBS is 30 chosen on the basis of its ability to support ES cell growth with low levels of differentiation (i.e., only rare individual cells undergo differentiation. The ES cells are grown in an atmosphere of 5-10% CO<sub>2</sub>, at 37°C

II. ROUTINE PASSAGE

ES cells must be passaged frequently to prevent the colonies from growing too large and differentiating. This is achieved by splitting the cells at a ratio of 1:10 to 1:40, every two to four days.

5

EXAMPLE 12

Genetic Manipulation of Cells

The general procedures set out in this Example provide guidelines that are readily adaptable to individual experimental situations that might employ, for 10 example, different cell lines or equipment supplied by different manufacturers. This Example also provides specific procedures used and results obtained in generating a set of mouse ES cell lines in which the  $\alpha$  1-3 galactosyltransferase gene was disrupted by homologous. 15 recombination. The general procedures provided in this Example are adapted for mouse ES cells. However, the procedures are substantially similar for porcine ES cells.

- I. INTRODUCTION OF DNA INTO ES CELLS BY ELECTROPORATION
  - 20 A. Coat required number of plates with 0.1% gelatin (in PBS or Milli-Q water). (Usually 2 X 6 well plates and 8 well plate)
    - B. Thaw  $10^7$  embryonic fibroblasts into DMEES (equivalent to ES Cell Medium); inactivate by irradiating 25 at 3000 Rad.
      - C. Count irradiated cells, spin down and resuspend in DMEES to  $10^6$  cells/ml.
      - D. Aspirate gelatin from plates and plate cells at: 7 X  $10^5$  cells/well (6 well plate) in 2.5ml medium; 7 30 X  $10^4$  cells/well (24 well plate) in 1 ml medium. Incubate at 37°C, 5-10% CO<sub>2</sub> for 3 - 4 hr.
    - E. Wash ES cells in 5 ml (250 ml flask) PBS-EGTA and let sit at room temperature for 4 min.

F. Remove PBS, add 5 ml trypsin (CSL) and leave at room temperature for 2 - 4 min. Wash down cells, add 10 ml DMEES and count. Approximately  $5 \times 10^6$  to  $2 \times 10^7$  ES cells are needed for experiments.

5 G. Centrifuge cells and resuspend in 10 ml PBS. Centrifuge again and resuspend in 540  $\mu$ l PBS. Dilute 50  $\mu$ l into 10 ml DMEES and culture to determine plating efficiency.

H. Add 5 - 10  $\mu$ g DNA to cells in 10  $\mu$ l PBS (total 10 volume, 500  $\mu$ l) and transfer to sterile electroporation cuvette (e.g. Biorad).

I. Electroporate at 0.22 kV, 500  $\mu$ FD (time constant should be ~8.4). This is achieved using a Biorad Gene Pulser unit (Biorad Catalogue No. 1652078) 15 with capacitance extender (Biorad Catalogue No. 1652087), or similar device.

J. Resuspend in 10 ml DMEES with constant pipetting to break up clumps of DNA from lysed cells.

K. Centrifuge cells and resuspend in 5ml DMEES.

20 L. Take 50  $\mu$ l, add 50  $\mu$ l trypan blue solution and count for viability.

M. Culture by dilution plating to determine plating efficiency.

## II. SELECTION CONDITIONS

25 ES cells that do not express a neomycin resistance gene are selectively killed by treatment with G418 at 200-500  $\mu$ g per ml of medium. Antibiotic- containing medium is changed daily. A population of cells that has not been electroporated also is treated in order to see 30 how genuinely sensitive cells respond to the G418 treatment. After 6 to 10 days, cells resistant to the antibiotic will be evident as healthy colonies. These cells will have been transformed by the targeting construct and can be screened for homologous

recombination (i.e., screened for gene targeting versus random integration).

Resistant colonies are picked from the selection dish with a mouth pipette and dispersed into a single cell suspension. Half of these cells are frozen away while the other half is expanded and used to determine whether or not homologous recombination has occurred. If the colonies are small, it is sometimes preferable to expand the whole colony in a 24 well dish, and then to freeze half while further expanding the other half for genetic analysis.

### III. PICKING ES CELL COLONIES FOR GENETIC ANALYSIS AFTER SELECTION

#### A. Method 1: Freezing Half Colonies

- 15 1. The day before colony picking:
  - a) Coat required number of plates with 0.1% gelatin (in PBS). Two plates per 24 colonies to be picked: one plate is for freezing and one plate is for clone expansion. Start with 20 X 24 well plates.
  - b) Count irradiated fibroblasts, spin down and resuspend in DMEES.
  - c) Aspirate gelatin from 10 plates and plate  $\sim 10^5$  (can use as few as  $5 \times 10^4$ ) cells/well in 1ml DMEES. Incubate at 37°C, 10% CO<sub>2</sub> overnight (or a minimum of 1 h).
  - 25 d) Aspirate the gelatin from the other 10 plates.
2. On the day of colony picking:
  - a) Change medium on ES cells before and regularly during picking (to remove floating cells).
  - 35 b) Pull plugged pasteur pipettes. Use a fresh pipette after each 24 colonies. The desired tip is about half a colony in diameter, with the constriction over 1-2cm. The tip

should be perpendicular and neat.  
Note: after drawing the pipette, rub  
the glass at the desired break point  
with freshly drawn glass, then bend.)

- k) Seal in ziplock bag or plastic wrap and place in -70°C freezer in an equilibrated styrofoam box. Interleave the plates with styrofoam sheet.
- l) Incubate expansion plates until there are sufficient cells for genotype analysis.

A. Method 2: Freezing after expansion to 24 wells.

1. The day before colony picking:
  - a) Coat required number of plates with 0.1% gelatin (in PBS). Start with 10 X 24 well plates.
  - b) Count irradiated fibroblasts, spin down and resuspend in DMEES.
  - c) Aspirate gelatin from the plates and plate  $\sim 10^5$  cells/well in 1ml DMEES. Incubate at  $37^{\circ}\text{C}$ , 10%  $\text{CO}_2$  overnight (or a minimum of 1 h).
2. On the day of colony picking:
  - a) Pick colonies as described for half colonies (method 1, above) but instead of dividing the cell suspension between freezing and expansion plates, the entire cell suspension goes into the expansion plate.
  - b) After 3-4 days (with daily medium changes) the cells will have grown sufficiently to be frozen. Working one plate at a time (with practice two can be handled), aspirate medium from each well. Flood with PBS/EGTA for 4 minutes. Meanwhile, set up pipette tips to fit alternate channels of a twelve channel multipipettor. Aspirate PBS.
  - c) Add 100  $\mu\text{l}$  trypsin (using multipipettor and alternate channels) and leave at room temp. for 2 min.

#### IV. THAWING OF ES CELL CLONES FROZEN IN 24-WELL PLATES

Cells that have been identified to have the desired genetic alteration are recovered from a duplicate plate frozen at -70°C. The plate is taken to the laminar flow hood and removed from the plastic bag. Each well is filled with warm medium, and feeder cells are added to the well(s) of interest. The plate is placed in a 37°C incubator for 60 min., then the medium is replaced. Colonies will appear after two or three days. These colonies are expanded for establishment of new frozen stocks, and tested for 1) karyotype analysis; 2) confirmation of the desired genetic alteration; 3) mycoplasma infection; and 4) ability to form chimeras.

EXAMPLE 13

Production Of Mouse ES Cell Knockouts Using  
The pNEOαGT10.8B Construct

I. TRANSFORMATION

5 A total of  $1 \times 10^7$  E14 ES cells was electroporated with  $5 \mu\text{l}$  of  $1 \mu\text{g}/\mu\text{l}$  pNeoαGT10.8B DNA (linearized by XbaI digestion) (see Example 9 and Figure 17). Electroporation was carried out in  $600 \mu\text{l}$  in a wide cuvette at  $25 \mu\text{F}$ ,  $350\text{V}$  for  $0.5\text{msec}$ . Cells were recovered in  $6\text{ml}$  ES complete  
10 medium and plated into  $6 \times 100\text{mm}$  petri dishes, each containing a feeder layer of Neo<sup>R</sup> STO cells.

Cells were cultured in ES complete medium for 3 days and then medium containing  $200\text{--}350 \mu\text{g}/\text{ml}$  G418 was substituted. This medium was changed every second day.

15 After 9 days, individual Neo<sup>R</sup> colonies were sufficiently large to be identified and recovered. Colonies were picked in  $20 \mu\text{l}$  PBS and  $20 \mu\text{l}$  of trypsin solution were added. Forty  $\mu\text{l}$  of 60% BRL conditioned medium in ES complete medium were then added. Aliquots of  $40 \mu\text{l}$  were  
20 transferred to single wells of each of two 24-well plates. One plate contained a feeder layer of STO cells in  $100 \mu\text{l}$  ES complete medium.  $140 \mu\text{l}$  of 2x DMSO freezing mix was added to this plate, which was stored at  $-80^\circ\text{C}$ . Each of the wells of the second 24-well plate contained  
25  $1\text{ml}$  of 60% BRL conditioned medium in ES complete medium. This plate was incubated at  $37^\circ\text{C}$  until the colonies were confluent.

II. CONFIRMATION OF HOMOLOGOUS RECOMBINATION

Medium was aspirated off confluent colonies and  
30  $400 \mu\text{l}$  lysis buffer (10mM Tris pH 7.8, 100mM NaCl, 1mM EDTA, 1% SDS, and  $500 \mu\text{g}/\text{ml}$  Proteinase K) added. The cells were lysed at  $37^\circ\text{C}$  overnight, extracted with  $400 \mu\text{l}$  1:1 phenol/chloroform and transferred to Eppendorf tubes containing  $1\text{ml}$  95% ethanol and  $0.2\text{M}$  NaAc. DNA was

pelleted by centrifuging at 13,000 rpm in an Eppendorf centrifuge, the pellet washed twice with 80% ethanol and redissolved in 30 $\mu$ l water.

Southern analysis (see, e.g., Sambrook et al., 5 supra) was used to identify ES cell clones where homologous recombination had occurred at the 3' end of the construct. Aliquots of 15 $\mu$ l of DNA were digested with 20 units of the restriction enzyme BglII according to the manufacturer's recommendations. After incubation 10 at 37°C overnight, the DNA was electrophoresed through a 0.8% agarose gel (in a Tris acetate, EDTA buffer) at 1-2V/cm overnight, using 750ng of HindIII-digested lambda DNA as markers. The DNA was transferred to a Zetaprobe nylon membrane using a Hybaid vacublotter at a vacuum of 15 80cm Hg for 1 hour.

The membrane was prehybridised in a Hybaid hybridization bottle in 10ml of the following hybridization mix for 3 hours at 65°C:

20 0.25M Na<sub>2</sub>HPO<sub>4</sub> pH 7.2  
7% SDS  
1mM EDTA  
100 $\mu$ g/ml salmon sperm DNA  
10% PEG

Radioactively labeled probe DNA was prepared using a 25 BRESATEC gigaprime oligo labeling kit (Cat. No. GPK-1) according to the manufacturer's recommendations. Approximately 50ng of a 0.7kb EcoRI/XmnI DNA fragment from beyond the 3' terminus of the construct pNeo $\alpha$ GT10.8B (see Example 9 and Figure 17) were labeled with <sup>32</sup>P-dATP 30 to a specific activity of 5x10<sup>8</sup> cpm/ $\mu$ g. The denatured probe was added to the prehybridising membrane in the Hybaid bottle and incubated overnight at 65°C.

The membrane was removed from the Hybaid bottle, rinsed with 0.5xSSC, 0.1% SDS prewarmed to 65°C, and then 35 washed 2-3 times with 0.1xSSC, 0.1% SDS at 65°C for 30 min each wash. Excess moisture was then blotted from the

membrane, the membrane wrapped in plastic wrap and exposed to a phospho-imager screen for 16 hours up to 3 days. The image was visualized on an Imagequant phospho-imager.

5       Results are shown in Figure 18, which is a Southern blot of DNA from 15 ES cell lines probed with the diagnostic 0.7kb EcoRI/XmnI DNA fragment described above and in Example 9. The 6.4kb band, diagnostic for a homologous recombination event in the  $\alpha$  1-3  
10 galactosyltransferase gene ( $\alpha$  1-3 Gal T) (see Example 9), is seen in 6 of the 15 ES cell lines examined. All of the 6 knockout cell lines appeared to be heterozygous for the inactivated allele since the 8.3kb band, diagnostic for the uninterrupted  $\alpha$ -1,3-Gal T gene (see Example 9),  
15 was also present in all six lanes.

Two cell lines, designated hereinafter "8D1" and "7C2," were chosen for further analysis. Cell lines 8D1 and 7C2 were identified by Southern analysis to contain an  $\alpha$ -1,3-Gal T allele where homologous recombination had  
20 occurred at the 3' boundary of the construct.

Long range PCR was then used to determine whether or not homologous recombination had occurred at the 5' boundary of the construct within these cell lines. Two sets of primers were used in separate PCR experiments:

25   1) Wild-type primers:-

MGT-KOex8F and MGT-KOR1 span the intron between exons 8 & 9, and amplify a 5.5 kb fragment from the wild-type  $\alpha$ -1,3-Galt gene (Figure 19)

SEQUENCES:

30       MGT-KOex8F

5' TGCTGGAAAAGTACTACGCCACACAGAACTCA-3'

(SEQ ID NO: 14)

(Nucleotides 1014-1046 in Figure 4)

MGT-KOR1

35       5' AGCCAGAGTAATAGTGTCAAGTTCCATCACAA-3'

(SEQ ID NO: 15)  
(Nucleotides 1779-1811 in Figure 4)

2) Knockout primers:-

MGT-KOex8F and MGT-KONeoR span exon 8 to the Neo<sup>R</sup> gene cassette in the "knock-out" allele and amplify a 5.5 kb fragment from the knocked out allele (Figure 19)

SEQUENCE:

MGT-KONeoR

5'-GCCACACGCGTCACCTTAATATGCCAAGTGGAC-3'

10 (SEQ ID NO: 16)

(Nucleotides 323-355; Figure 16)

Each reaction contained ~100 ng genomic DNA as template in a reaction volume of 50 $\mu$ l and contained 25mM Tris HCl (pH9.1), 16mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 250  $\mu$ M dNTPs, 3.5 mM 15 MgCl<sub>2</sub>, 100 ng each primer, 2 units Taq polymerase and 0.025 units Pfu polymerase. The reactions were heated at 94°C for 1 min, then 45 cycles of 94°C for 15 sec, 68°C for 6 min, followed by a single step of 72°C for 10 min. Genomic DNAs from putative "knock-out" ES cell lines from 20 CBA/C mice (homozygous for the wild-type  $\alpha$ -1,3-Gal T allele) were amplified in separate reactions using each set of primers. A 10 $\mu$ l aliquot of each PCR was analyzed by Southern blotting (Sambrook et al., 1989).

The results are illustrated in Figure 20:

25 Knockout primers:-

A 5.5 kb fragment that hybridized to the 1.3 kb Neo<sup>R</sup> gene cassette (Figure 16) was generated from 7C2 DNA (Figure 20; lane 4) and 8D1 DNA (not shown). This band was not generated from CBA/cDNA (Figure 20; 30 lane 3).

Wild-type primers:-

A 5.5 kb fragment that hybridized to the  $\alpha$ -1,3-Gal T gene probe (isolated by Sal I digestion of p $\alpha$ GT-S4.0) was generated from 7C2 and CBA/cDNA's (Figure

20; lanes 1 and 2 respectively) and 8D1 DNA (not shown). This product did not hybridize to the Neo<sup>R</sup> gene probe.

These results demonstrate that homologous recombination  
5 had occurred at the 5' boundary of the construct in cell lines 8D1 & 7C2.

EXAMPLE 14

Generation of Animals Carrying an ES Cell Genome

The procedures provided in this Example are  
10 adapted for mouse ES cells. However, the general strategy is substantially the same for porcine ES cells and PGC's.

I. PREPARATION OF ES CELLS FOR INJECTION

ES cells are split into wells of a 24-well dish at cell densities of 1:2, 1:4, 1:8 and 1:16, relative to  
15 the initial density, two and three days before injection. The most vigorous and least differentiated cultures are chosen on the basis of morphology.

II. EMBRYO INJECTION AND PRODUCTION OF CHIMERIC MICE

Mouse embryos are collected from either  
20 superovulated or naturally mated female mice, approximately 3.5 days after mating. After overnight culture in M16 medium (Bradley, Production and Analysis of Chimaeras. In Teratocarcinomas and Embryonic Stem Cells a Practical Approach (E.J. Robertson, ed.) IRL Press, Oxford, pp. 113-25 52 (1987)), those that have cavitated to form blastocysts are microinjected with about 12 to 20 ES cells. This microsurgical procedure is performed with instruments drawn from capillary glass, and injection is controlled with micrometer syringe-based hydraulic devices. A differential  
30 interference contrast-equipped inverted microscope is used to view the procedure.

After injection, blastocysts are transferred to the uterus of pseudopregnant female mice. Chimeric mice are identified by coat color contribution by the ES cells. Chimeric mice show agouti coat colour derived from the host 5 blastocyst, and chinchilla contributed by the ES cells.

Chimeric mice were generated from ES cells carrying the interrupted  $\alpha$ -1,3-Gal T allele (including 8D1, 7C2 cells) by injection into C57B1/6J x CBA F2 blastocysts. The ability of individual chimaeric mice to transmit the ES 10 cell characteristics through the germ-line was estimated by glucose phosphate isomerase (Gpi) analysis of sperm (Bradley, supra, (1987)); Mann et al., J. Reprod & Fert. 99, 505-512 (1993). Glucose phosphate isomerase catalyses the interconversion of glucose-6-phosphate to fructose-6- 15 phosphate. Mice have a single structural Gpi locus with two main alleles Gpi 1A and Gpi 1B. Gpi 1A codes for protein which appears as a slow cathodically migrating band during electrophoresis and occurs in strains such as BALB/c and C129. (The ES cells used here were derived from strain 20 129 mice). Gpi 1B determines an enzyme that moves faster than Gpi 1A and occurs in the wild and in strains such as C57 and CBA (used here to derive host blastocysts).

Heterozygotes have the two parental bands plus an intermediate band which indicates the dimeric structure of 25 the enzyme. Multiple electrophoretic forms occasionally observed are due to oxidation of sulphydryl groups and not due to tissue-specific expression. In chimaeric mice, the ratio of Gpi 1A (strain 129-derived) to Gpi 1B (derived from the host blastocyst) indicates the proportion of cells 30 with the ES cell genotype within different tissues. The appearance of Gpi 1A (derived from the ES cells) in the sperm suggests that the mouse is able to transmit the ES cell genotype through the germ-line.

III. GENERATION OF MICE HOMOZYGOUS FOR THE GENETIC CHANGE  
INTRODUCED INTO THE ES CELLS.

Chimeric mice with sperm derived from ES cells were mated to BALB/c mice. Offspring with the 129/Ola X BALB/c 5 genotype (i.e. heterozygous for the ES cell genotype) are grey. Half of these grey mice were expected to carry the interrupted allele. Mice heterozygous for the interrupted allele were identified by PCR analysis of genomic DNA obtained from blood.

10 To generate mice homozygous for the inactivated  $\alpha$ -1,3-Gal T gene, the heterozygous mice were mated to each other. One quarter of the offspring were expected to be homozygous for the interrupted gene. Homozygotes were identified by PCR analysis of genomic DNA obtained from blood. The PCR 15 strategy was based on the insertion of a Neo<sup>R</sup> gene in the Sal I site of exon 9 of the  $\alpha$ -1,3-Gal T gene (Figure 13).

Wild-type primers:-

E9F: 5'TCAGCATGATGCGCATGAAGAC 3'

(SEQ ID NO: 17)

20 (homologous to sequence about 40 to 60 bp 5' to the Sal I site of exon 9, corresponding to nucleotides 1257-1278; Figure 4)

E9R2: 5'TGGCCCGTGGTAGTAAAAA 3'

(SEQ ID NO: 18)

25 (homologous to a region about 175 to 195 bp 3' to the Sal I site of exon 9, corresponding to nucleotides 1511-1492; Figure 4)

The expected fragment size generated from the wild-type allele is 255 bp (Figure 21). These primers also can 30 potentially generate a 1596 bp PCR fragment from the interrupted allele. In practice this fragment was not generated when both the wild-type and interrupted alleles

were present, probably because the smaller 255 bp product is amplified preferentially.

Knock-out primers:-

NeoF1: 5' TCTTGACGAGTTCTTCTGAG 3'

5 (SEQ ID NO: 19)

(corresponding to nucleotides 1170-1189; Figure 16)

E9R2: (the same primer described above to detect the wild-type allele)

The expected fragment size is 364 bp (Figure 21).

10 Mice were grown to weaning age and bled from the tail.

Sodium Heparin was added to about 10 U/ml. PCR amplification was conducted on 1  $\mu$ l of heparinised blood ( $\sim 10^4$  nucleated cells) in a 50 $\mu$ l reaction volume containing 100 mM Tris-Acetate pH 8.8, 3.5 mM MgCl<sub>2</sub>, 0.2mM dNTPs, and 15 2 units Tth DNA polymerase. Each reaction contained both the wild-type and knock-out primers at a concentration of 2ng/ $\mu$ l for each primer. To ensure that Tth polymerase was not inhibited by heparinized blood, each reaction was performed in duplicate.

20 One of the reactions was spiked with two DNA samples:

i) 10 fg (~600 molecules) of linearized KO plasmid pNeo $\alpha$ GT10.8B.

ii) 1 fg (~1000 molecules) of a 983 bp RT-PCR product that includes Exon 9.

25 The other reaction was not spiked. Thus, two separate PCR reactions were set up for each blood sample. In addition, control PCR reactions with no genomic DNA template and with or without spikes were conducted. Each reaction mix was heated at 94°C for 3 min., then incubated for 40 cycles at 30 94°C for 40 sec., 53°C for 40 sec., and 72°C for 40 sec. Aliquots of 5  $\mu$ l of each reaction mix were electrophoresed on a 3% agarose gel, and DNA fragments were visualized on a UV light box after staining with ethidium bromide. HpaII-digested pUC19 plasmid DNA was used for markers.

Results of the PCR analysis for three mice, and a "no DNA" control, are shown in Figure 22. For mouse #42, the KO primers generated a 364 bp band in the + spike reaction only. The wild-type primers generated a 255 bp band in the 5 + spike and - spike reactions. These results demonstrate that mouse #42 is homozygous for the wild-type allele. For mouse #43, the wild-type primers generated a 255 bp band in the + spike reaction only. The KO primers generated a 364 bp band in the + spike and - spike reactions. These 10 results demonstrate that mouse #43 is homozygous for the interrupted allele. For mouse #44, the KO primers generated a 364 bp band in the + spike and - spike reactions. The wild-type primers generated a 255 bp band in the + spike and - spike reactions. These 15 results demonstrate that mouse #44 is heterozygous for the interrupted allele. In the control PCR reactions, no product was evident when template was not included. PCR products of 364 bp and 255 bp were evident when pNeo $\alpha$ GT10.8B and Exon 9 RT-PCR DNA were the only templates 20 included in the control reactions.

EXAMPLE 15

Characterization of Homozygous Knockout Mice

I. ABSENCE OF Gal T mRNA IN Gal T KNOCKOUT MICE

A. RNA Isolation

25 Total RNA was extracted using the RNAzol<sup>TM</sup>B kit (BIOTECX Laboratories, Inc., 6023 South Loop East, Houston, Texas 77033, USA.), supplied by Bresatec. This extraction procedure is based on the method described by Chomczynski et al., Anal. Biochem. 162: 156-159 (1987), and involves 30 homogenization in a guanidinium/phenol solution, a chloroform extraction, 2 isopropanol precipitations, and 75% EtOH washes. The RNA was stored as an EtOH precipitate at -20°C and quantitated by measuring absorption at

wavelength 260 nm in water. The integrity and quantitation was confirmed by electrophoresis in agarose/formaldehyde gels. Sambrook et al. Molecular Cloning. A Laboratory Manual. Second Edition. (1989)

5        B. RT-PCR

First strand cDNA synthesis involved:

- annealing 2 $\mu$ g of total RNA from kidney, heart or liver with 120ng oligo dT primer (Gibco BRL, M-MLV Reverse Transcriptase Kit) at 65°C for 5 minutes in 5 $\mu$ l of 10 mM Tris-HCl, 1mM EDTA (pH8).
- 10        - reverse transcription at 37°C for 1-2 hours in a final reaction volume of 20 $\mu$ l utilizing the M-MLV Reverse Transcriptase Kit(Gibco BRL). Each reaction contained 5mM DTT, 0.1 $\mu$ g/ $\mu$ l BSA, 1mM dNTPS, 40 U of human placental RNase Inhibitor (Bresatec), 200U of M-MLV Reverse Transcriptase and the associated RTase buffer at 1X concentration.

15        C. PCR Analysis of cDNA

20         $\alpha$ -1,3-Gal T cDNA was detected by PCR amplification of oligo dT-primed cDNA template. Failure to generate this PCR fragment, in conjunction with the control PCR results, indicated that  $\alpha$ -1,3-Gal T mRNA was absent from the RNA preparation. To demonstrate that the  $\alpha$ -1,3-Gal T primers supported amplification of the  $\alpha$ -1,3-Gal T template, each reaction was assembled in duplicate, and one of the reactions was spiked with 0.1 fg (~100 molecules) of a 983 bp mouse  $\alpha$ -1,3-Gal T cDNA product (generated by primers 7F and mGT-3UR, spanning exon 7 to the 3' untranslated region). As a second control to demonstrate that cDNA synthesis had occurred, a ferrochelatase PCR fragment was generated from the cDNA template.

25        1. Primers:

30        Primers to detect  $\alpha$ -1,3-Gal T cDNA:

7F: 5'-TGGAGATCGCATTGAAGAGC 3'  
(SEQ ID NO: 20)  
(corresponding to nucleotides 889-911  
within exon 7 (Figure 4))  
5 9R2: 5'-TGGCCGCGTGGTAGTAAAAA 3'  
(SEQ ID NO: 21)  
(corresponding to nucleotides 1492-1511  
within exon 9 (Figure 4))

10 Primers 7F and 9R2 were expected to generate a  
fragment of ~619 bp (Figure 23) from the cDNA  
template. These primers will not generate a fragment  
from genomic DNA possibly present in the cDNA  
preparation, since the primers span two large introns.

15 mGT-3UR: 5'-GGGTTTGGTTTGATTGTT 3'  
(SEQ ID NO: 22)  
(corresponding to nucleotides 1866-1888  
within the 3' untranslated region;  
Figure 4).

20 This primer was used with primer 7F to generate the  
DNA fragment used in the control spike PCRs.

Primers to detect mouse ferrochelatase cDNA (EcoRI  
linkers, underlined):

25 FC-F: 5'-CTGAATTCATGTTAACACATGGGAGGCC 3'  
(SEQ ID NO: 23)  
(corresponding to nucleotides 215-235,  
Taketani et al., J. Biol.Chem. 265:  
19377-80 (1990)).

30 gFC-R: 5'-CTGAATTCTGCCCACTCCCTGCCGATG 3'  
(SEQ ID NO: 24)  
(corresponding to nucleotides 888-908,  
Taketani et al., J. Biol.Chem. 265:  
19377-80 (1990)).

These primers were expected to generate a 709 bp fragment (Figure 23). These primers will not generate a fragment from genomic DNA possibly present in the cDNA preparation, since the primers span five introns.

Reaction volumes were 50  $\mu$ l, consisting of 4  $\mu$ l of the first strand cDNA synthesis reaction, 100 ng of each primer, 2 mM MgCl<sub>2</sub>, 0.3 mM dNTPS, 2U of Taq-Polymerase (Bresatec) and Taq reaction buffer (Bresatec) at 1X concentration. Reactions were heated at 94°C for 2 min, then 29 cycles of 94°C for 15 sec, 58°C for 30 sec and 72°C for 1 min followed by single steps of 72°C for 4 min and 4°C for 5 min. A 10  $\mu$ l aliquot of each PCR was electrophoresed on a 2% agarose gel and DNA fragments were visualized on a UV light box after staining the gel with ethidium bromide.

Figure 24 shows the PCR fragments generated from RNA isolated from kidney (K), heart (H) and liver (L) of a wild-type mouse, and mice heterozygous or homozygous for the interrupted  $\alpha$ -1,3-Gal T allele. Figure 24(i) shows that the 709 bp ferrochelatase fragment was generated from each of the cDNA preparations, indicating that cDNA template was produced from the reverse transcription reaction, and was available for the  $\alpha$ -1,3-Gal T gene primers. The 619 bp  $\alpha$ -1,3-Gal T fragment was present in each of the reactions spiked with the 983 bp  $\alpha$ -1,3-Gal T cDNA product (Figure 24(ii)), indicating that amplification of the  $\alpha$ -1,3-Gal T cDNA (spike) template had occurred.

In the reactions that were not spiked (Figure 24 (iii)), the 619 bp  $\alpha$ -1,3-Gal T fragment was detected in cDNAs synthesized from the wild-type and heterozygous RNAs. This indicates that  $\alpha$ -1,3-Gal T mRNA is present in the kidney, heart and liver of the wild-type and heterozygous mice. The 619 bp fragment was not detected in the unspiked homozygous KO reactions, indicating that  $\alpha$ -1,3-Gal T mRNA is not synthesized in the homozygous KO mice.

II. TEST FOR EXPRESSION OF THE GAL EPITOPE IN HOMOZYGOUS KNOCKOUT MICE USING ANTI-GAL ANTIBODIES WITH FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

A. Solutions

5 Solutions 1 to 5 are 10x isotonic.

1. 1.68M NaCl (948.21g/l) Dry salts overnight in hot oven before weighing

2. 1.68M KCl (125g/l) Dry salts overnight in hot oven before weighing

10 3. 1.12M CaCl<sub>2</sub> (165g/l CaCl<sub>2</sub>2H<sub>2</sub>O) Dry salts overnight in hot oven before weighing

4. 1.68M MgSO<sub>4</sub> (414g/l MgSO<sub>4</sub>7H<sub>2</sub>O) Do not dry in hot oven

5. Potassium phosphate buffer pH 7.2:

15 a) 1.68M KH<sub>2</sub>PO<sub>4</sub> (229 g/L)

b) 1.12M K<sub>2</sub>HPO<sub>4</sub> (226 g/L K<sub>2</sub>HPO<sub>4</sub> 3H<sub>2</sub>O or 195 g/l K<sub>2</sub>HPO<sub>4</sub>)

Potassium phosphate buffer is prepared by mixing together equal volumes of solutions a) and b). To 20 pH the buffer, remove a small sample, dilute 1:50 and read on pH meter.

6. Hepes buffer 1M (CSL, Melbourne Australia)

7. KDS BSS:

Add stock solutions in the following order to 25 double-distilled water (DDW):

Stock	Ratio of Solutions
DDW	1210
NaCl	121
KCL	3
CaCl <sub>2</sub>	3
MgSO <sub>4</sub>	1
Potassium phosphate buffer	2
Hepes	20

Filter sterilise, store at 4°C

8. KDS/BSS/2%HSA/0.02% azide:

	KDS/BSS	244.5ml
	Human serum albumin	5ml
	(CSL, Melbourne, Australia)	
5	10% Na azide in MT-PBS	0.5ml

9. FITC dilution: Dilute 7.5ul FITC-IgG to 600ul with KDS/BSS

10. Red cell lysis buffer:

0.168M NH<sub>4</sub>Cl in double distilled water

10 11. 4% paraformaldehyde (PFA)

Solutions:

A. NaH<sub>2</sub>PO<sub>4</sub> 2H<sub>2</sub>O 22.6 g/L  
B. NaOH 25.2 g/L

C. 40% paraformaldehyde:

15 1) 4 g paraformaldehyde (BDH, Kilsyth, Australia, #29447) dissolved in 10ml double distilled water. Heat 70°C 2 hours on stirrer in fume hood and a few drops of 2M NaOH are added until the solution becomes clear.

20 2) 0.54 g glucose is then added.  
3) Store RT in light proof bottle.

D. Add together 83 ml of A + 17 ml of B.

25 E. Final 4% PFA fixative solution: 90 ml of D + 10 ml of C. pH 7.4 - 7.6; adjust pH with 1M HCl.

12. Hanks Balanced Salt Solution (Ca and Mg free) (HBBS):

30	KCL	400mg
	KH <sub>2</sub> PO <sub>4</sub>	60mg
	NaCl	8g
	NaHCO <sub>3</sub>	350mg
	Na <sub>2</sub> HPO <sub>4</sub> 2H <sub>2</sub> O	68mg
	Glucose	1g
35	H <sub>2</sub> O	to 1 liter

adjust to pH 7.0; filter sterilize

13. Sheep antihuman IgG and IgM fluorescein isothiocyanate (FITC) F(ab)2 fragments (Silenus, Hawthorn, Australia):

B. Methods

1. Eye bleed mice, collect 300-400ul into pre-chilled Ependorf tube, store on ice, add EDTA 20mg/ml to give final concentration of 2mg/ml.
- 5 2. Transfer blood (including appropriate human controls) to 10ml plain tube and add 10ml red cell lysis buffer (0.168M NH<sub>4</sub>Cl) pre-warmed to 42°C; incubate for several minutes or until cells have lysed.
- 10 3. Pellet cells by centrifugation (800 x g, 7 min, 4°C).
4. Resuspend cells in 10ml KDS/BSS/2% HSA/0.02% NaN<sub>3</sub>
5. Pellet cells as above; repeat steps 4 & 5.
6. Resuspend cells in 1000ul KDS/BSS/2% HSA/0.1% NaN<sub>3</sub>; transfer aliquots to V bottom FACS tubes.
- 15 7. Pellet cells as above.
8. Resuspend cells in 100ul KDS/BSS/2% HSA/0.1% NaN<sub>3</sub>
9. Add 50ul of purified anti-GAL antibody (see Example 1, above), normal human serum (NHS) or HBBS/2% HSA/0.1% NaN<sub>3</sub> and incubate 45 min.
- 20 10. Add 2ml KDS/BSS/2% HSA/0.02% NaN<sub>3</sub>; centrifuge cells as above.
11. Add 50ul of a 1:80 dilution of sheep antihuman IgG or IgM FITC F(ab)2 fragment (Silenus).
- 25 12. Add 2ml KDS/BSS/2% HSA/0.02% NaN<sub>3</sub>; centrifuge cells as above.
13. Resuspend cells in 300ul KDS/BSS/2% HSA/0.02% NaN<sub>3</sub>.
- 30 14. Transfer samples to plastic round-bottom FACS tubes and add 3 ul of propidium iodide (100ug/ml); samples are now ready for analysis; keep on ice.
15. Analyse on Beckman FACS scan using peripheral blood lymphocyte settings.

C. Results

The results of these experiments are given below:

	median channel fluorescence (log scale)	peak channel fluorescence (log scale)	
5	MOUSE 129 (Normal) PBL + FITC anti- IgG alone (neg. control)	9	9
10	MOUSE 19 PBL (wild type) GAL IgG	197	286
15	MOUSE 21 PBL (Gal KO) GAL IgG	22	15
20	MOUSE 129 (Normal) PBL + FITC anti- IgM alone (neg. control)	7	1
25	MOUSE 19 PBL (wild type) GAL IgM	185	167
30	MOUSE 21 PBL (Gal KO) GAL IgM	34	18
	MOUSE 129 PBL (normal) PBL + FITC IgG alone (neg. control)	8	9
	MOUSE 129 PBL (normal) GAL IgG	120	328
	MOUSE 9 PBL (Gal KO) GAL IgG	10	9

The results of human anti-Gal binding to human peripheral blood lymphocytes (negative control) are not shown but were negative. These experiments demonstrate that human anti-Gal (IgG and IgM) antibodies bind to peripheral blood cells of the homozygous  $\alpha 1,3$  galactosyltransferase knockout mice (mouse 21 and mouse 9) very weakly if at all. This confirms the expected lack of the galactose  $\alpha 1,3$  galactose (GAL) epitope in

such mice. In contrast, peripheral blood cells of normal mice (mouse 129 and mouse 19) of the same strain display clear binding of anti-Gal antibodies.

5        III. TEST FOR EXPRESSION OF THE GAL EPITOPE IN HOMOZYGOUS  
KNOCKOUT MICE USING IB<sub>4</sub> LECTIN WITH FACS

IB<sub>4</sub> Lectin has an exclusive affinity for terminal  $\alpha$ -D-galactosyl residues, and is demonstrated below to be useful for characterizing the knockout mice.

10        A. Solutions

1. 4% paraformaldehyde (see above)

2. Mouse Tonicity PBS (MT-PBS)

Na<sub>2</sub>HPO<sub>4</sub>            2.27g

NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O    0.62g

NaCl                8.7g

15        Make up to 1 liter with DDW

3. Dead Cell Removal Buffer (DCRB):

-4.5 g Sorbitol

-7.6 g Glucose monohydrate, (6.93 g if anhydrous)

20        -12.5 ml KDS/BSS

-Make up to 100 ml with DDW

-Filter, store at 4°C

-Open only under sterile conditions

4. KDS/BSS (Mouse Tonicity, Hepes Buffered

25        Balanced Salt Solution pH 7.2) (see above)

5. Red cell lysis buffer (see above)

6. KDS/BSS/2%HSA/0.02%azide (see above)

7. Hanks Balanced Salt Solution (Ca and Mg free) (see above)

30        B. Methods

1. Remove spleen, hold with curved forceps and collect splenocytes by injecting with a 27 gauge needle bent at 90°C, injecting (2.5 ml syringe) 100-200

ul buffer into the spleen two or three times. Using the flat surface of the bent needle massage cells out of holes made in spleen. Repeat injections and removal of cells until no cells remain in capsule.

5 2. Transfer splenocytes to 10ml tube and centrifuge to pellet cells (500xg, 7 min, 4°C).

10 3. Remove supernatant and add 3ml red cell lysis buffer pre-warmed to 42°C; incubate for several minutes or until cells have lysed. Underlay with 1ml HIFCS (heat inactivated fetal calf serum) and stand on ice 5 minutes. Top to 10ml with KDS BSS/10% HIFCS.

4. Centrifuge as above.

5. Resuspend cells in 3ml dead cell removal buffer; mix well with pipette.

15 6. Pass through a glass pipette plugged with cotton wool and collect cells into a 10ml tube. Don't force cells through, allow to drain under gravity.

7. Underlay cells with 1 ml BSS/10% HIFCS.

8. Centrifuge as above.

20 9. Remove supernatant.

10. Centrifuge as above; repeat steps 4 & 5.

11. Add 0.5 ml cold 4% paraformaldehyde (PFA).

12. Incubate on ice for 5 min with intermittent mixing.

25 13. Add 2 ml ice cold HBBS and centrifuge as above.

14. Repeat washings with 2ml and then 1ml HBBS.

30 15. Resuspend cells in 100ul KDS/BSS/2% HSA/0.1% NaN<sub>3</sub>; transfer to V bottom FACS tubes.

16. Add FITC IB4 lectin (Sigma, Cat. No. L 2895), 50ul at 20ug/ml, or 50ul HBBS; incubate on ice for 30 min.

35 17. Add 2ml KDS/BSS/2% HSA/0.1% NaN<sub>3</sub>; spin cells as above.

18. Resuspend cells in 300ul KDS/BSS/2%  
HSA/0.1% NaN3.

19. Transfer samples to plastic round-bottom  
FACS tubes; samples are now ready for analysis; keep on  
5 ice.

20. Analyse on FACS scanner using peripheral  
blood lymphocyte setting.

C. Samples

10 1. Mouse 129 splenocytes alone  
2. Mouse 129 splenocytes + IB4 lectin  
3. human PBL alone  
4. Human PBL + IB4 lectin

D. Results

Results of these experiments are given below:

	mean fluorescence channel (log scale)	median fluorescence channel (log scale)	peak fluorescence channel (log scale)
15	splenocytes alone (autofluorescence)	1	1
	mouse 19 (wild type) splenocytes	252	58
20	mouse 21 (KO mouse) splenocytes	3	2

25 The results demonstrate that IB<sub>4</sub> lectin binds  
spleen cells of the homozygous  $\alpha$ 1,3 galactosyltransferase  
gene targeted (Gal KO) mouse (mouse 21) very weakly if at  
all. This confirms the expected lack of the galactose  
 $\alpha$ 1,3 galactose (GAL) epitope in such mice. In contrast,  
peripheral blood cells of a normal mouse (mouse 19) of  
the same strain binds IB<sub>4</sub> lectin strongly.

IV. IMMUNOHISTOLOGICAL ASSESSMENT OF MOUSE TISSUES FOR  
THE PRESENCE OF THE GAL EPITOPE USING ANTI-GAL  
ANTIBODIES.

A. Reagents

5        1. TBS: Tris Buffered Saline  
            NaCl                    8g  
            KCl                    0.2g  
            Tris base            3g  
            - dissolve in 800ml distilled water. Adjust pH  
10 to 8.0 with 1 M HC1. Adjust volume to 1L. Sterilise by  
autoclaving. Store at RT.  
15        2. Blocking buffer:  
            - TBS + 2% bovine serum albumin (BSA) + 10%  
            rabbit serum:  
20        3. Peroxidase conjugates:  
            DAKO (Denmark) peroxidase (POD) conjugated to  
            rabbit anti-human IgG (fragment) and DAKO (Denmark)  
            peroxidase (POD) conjugated to rabbit anti-human IgM  
            (fragment).  
25        Conjugates were both separately pre-absorbed on 10%  
            mouse liver powder at 4°C overnight, then centrifuged at  
            18,000xg for 10 minutes in a Biofuge and then at 30 psi  
            for 30 min in a Beckman airfuge. Conjugated antisera  
            were diluted 1/50 in 2% blocking buffer (TBS + 2% BSA +  
25 2% rabbit serum) with 16% normal mouse serum.  
30        4. Mouse liver powder preparation:  
            As modified from Antibodies, a Laboratory  
            Manual Ed Harber and David Lane, Cold Spring Harbour  
            Laboratories (1988) p663:  
            a) Prepare a fine suspension of mouse liver  
            in mouse tonicity phosphate buffered saline (MT-PBS).  
            Mash liver through a sieve with a 5 ml plunger. Discard  
            any fibrous tissue. One gram of tissue should be  
            resuspended in approximately 1 ml MT-PBS.

b) Transfer the tissue/saline suspension to ice for 5 min.

c) Add 8 ml of acetone (-20°C) (Univar 6, Ajax Chemicals) for 10 minutes. Mix vigorously.

5 Incubate on ice for 30 minutes with occasional vigorous mixing.

d) Collect the precipitate by centrifugation at 10,000g (9,000 rpm in Sorvall RC-5B refrigerated superspeed centrifuge). Spin for 10 minutes.

10 e) Resuspend the pellet with fresh acetone (-20°C) and mix vigorously. Allow to sit on ice for 10 minutes.

f) Centrifuge at 10,000g for 10 minutes. Transfer the pellet to a clean piece of filter paper.

15 15 Spread the precipitate and allow to air-dry at room temperature.

g) After the pellet is dry, transfer it to an airtight container. Remove any large pieces that will not break into a fine powder. Dессіtate and store at 20 4°C. Yield as approximately 10-20% of the original wet weight. To use acetone powders, add to a final concentration of 1%. Incubate for 30 min at 4°C. Spin at 10,000g for 10 minutes. (13,000 rpm in Biofuge)

5. DAB/H<sub>2</sub>O<sub>2</sub>/Imidazole:

25 Peroxidase substrate: 3,3'-Diaminobenzidine tetrahydrochloride (DAB) (Sigma, Missouri)

- 1 tablet DAB (take out of fridge 10 min before use)

- 1 tablet urea H<sub>2</sub>O<sub>2</sub> (Sigma, Missouri)

30 - add to 15 ml tris HCL, pH 7.6 + 0.01M imidazole (0.0102g), (Sigma, Missouri)

- make up immediately before use

6. Tris HCL:

1.211g Tris in 200ml double distilled water pH

7.6

7. Animal serum sources:

5 Mouse and rabbit sera were obtained in-house  
(St. Vincent's Hospital, Dept, of Clinical Immunology).

Sheep serum was obtained from the University of  
Melbourne Veterinary Clinic and Hospital, Werribee,  
Australia.

10 8. Harris Haematoxylin:

Haematoxylin C.I. 75290 (BDII, Poole, U.K.

#34037) 10g

Absolute ethanol 200ml

Potassium alum 200g

15 double distilled water 2000ml

glacial acetic acid 80ml

Preparation:

1. Dissolve haematoxylin in absolute ethanol
2. Heat to dissolve alum in double distilled

20 water

3. Mix solution 1 and 2

4. Immediately before use add 80 ml 1% sodium  
iodate and 80 ml glacial acetic acid

9. Scott's Tap Water:

25 Sodium hydrogen carbonate 14 g

MgSO<sub>4</sub> 80 g

Tap water 4 litres

B. Methods

1. Cut 4 um sections of the relevant tissue on  
30 cryostat
2. Tissue should be free of cracks

3. Air dry slides for 30 min
4. Apply 10% blocking buffer at room temp in humidified chamber, 60 min
5. Remove blocking antibody with tissue made to fine point
6. Apply 1st antibody, anti-GAL, or 2% blocking buffer as control, 50ul, ensure no air bubbles and incubate at room temp in humidified chamber for 30 min
- 10 7. Wash off with Tris buffered saline (TBS) 3 times 2 minutes washes
8. Apply second antibody 1:50 peroxidase (POD) conjugated rabbit anti-human IgG and IgM (DAKO, Denmark); incubate 30 min at room temp in humidified chamber
- 15 9. Wash off with Tris buffered saline (TBS) 3 times 3 minute washes
10. Wash off with TBS as above
11. Incubate DAB/H<sub>2</sub>O<sub>2</sub>/imidazole for 10 minutes
- 20 12. Wash in water
13. Stain with haemotoxylin C - 10 seconds
14. Wash in water
15. Place in Scotts tap water for 15 seconds
16. Wash in water
- 25 17. Wash in absolute alcohol (x3) (Univar 214, Ajax chemicals)
18. Wash in absolute xylene (x3) (Univar 577, Ajax chemicals)
19. Coverslip using automatic coverslip machine (Tissue Tek)
- 30

Controls:

1. Buffer only + POD conjugated rabbit anti-human IgM (negative)

2. Buffer only + POD conjugated rabbit anti-human IgG (negative)
3. Human kidney (negative)
4. Pig renal cortex (positive)

5 Samples:

1. Mouse 129 SV (control) kidney
2. mouse 9 (Gal Knockout) kidney
3. mouse 21 (Gal Knockout) kidney

C. Results

10 KIDNEY

	GLOMERULI	ENDOTHELIUM	comments
15	MOUSE 129 anti-IgM	POSITIVE	POSITIVE
	MOUSE 9 anti-IgM	NEGATIVE	weak adventitial staining
	MOUSE 21 anti-IgM	NEGATIVE	weak adventitial staining
20	MOUSE 129 anti-IgG	POSITIVE	POSITIVE
	MOUSE 9 anti-IgG	NEGATIVE	NEGATIVE
	MOUSE 21 anti-IgG	NEGATIVE	NEGATIVE
	POD conjugated antibody alone	ALL NEGATIVE	ALL NEGATIVE

25 These results indicate that human anti-Gal IgG and IgM antibodies do not bind kidney tissue of the  $\alpha$ 1,3 galactosyltransferase gene targeted (Gal KO) mice (mouse 21 and mouse 9). This confirms that lack of the galactose  $\alpha$ 1,3 galactose (GAL) epitope in the gene targeted (KO) mice. In contrast, these antibodies react strongly with the endothelium of blood vessels and the glomeruli of a normal mouse of the same strain (129).

V. IMMUNOHISTOLOGICAL EXAMINATION OF MOUSE TISSUES  
USING IB<sub>4</sub> LECTIN

A. Reagents

1. Blocking buffer: TBS + 2% BSA + 10% sheep serum
- 5 2. FITC IB<sub>4</sub> (Sigma, Missouri, USA #L-2895)  
1 mg diluted in 1 ml HBBS to give stock solution, then dilute to final volume of 20 ug/ml in TBS + 2% BSA + 2% sheep serum
- 10 3. Peroxidase anti-FITC  
Boehringer anti-fluorescein POD Fab fragments; dilute 1/300 in 2% blocking buffer
4. DAB/H<sub>2</sub>O<sub>2</sub>/Imidazole - see above
5. Tris HCL - see above
- 15 6. Animal serum sources - see above
7. Harris Haematoxylin - see above
8. Scott's Tap Water - see above

- B. Methods
- 20 1. Preparation of Sections; same as Section 4B, steps 1-7 above.
2. Apply 50  $\mu$ l FITC conjugated IB4 (Sigma # 1-2894) 20  $\mu$ g/ml, incubate in a humidified chamber for 30 minutes.
- 25 3. Wash with TBS, 3 minutes (x3).
4. Apply 50  $\mu$ l peroxidase conjugated anti - FITC Fab fragments (Boehringer Mannheim), diluted 1-3--with TBS + 2% BSA + 2% sheep serum. Incubate for 30 minutes in humidified chamber.
- 30 5. Wash with TBS, 3 minutes (x3).
6. Processing for microscopy - same as Section IVB steps 14-22.

**Controls**

1. Buffer only + POD anti-FITC (negative)
2. Human kidney (negative)
3. Pig renal cortex (positive)

5      **Samples 1st Experiment**

1. Mouse 129 SV normal mouse heart liver kidney lung
2. mouse 6 wild type heart liver kidney lung
3. mouse 7 heterozygote KO heart liver kidney lung
4. mouse 9 homozygous KO heart liver kidney lung

10     **Samples 2nd Experiment**

1. mouse 19 wild type heart liver kidney lung
2. mouse 20 heterozygote KO heart liver kidney lung
3. mouse 21 homozygous KO heart liver kidney lung

C.     Results

15     **Kidney**

	GLOMERULI	ENDOTHELIUM
HUMAN	NEGATIVE	NEGATIVE
PIG	POSITIVE	POSITIVE
129 MOUSE	POSITIVE	POSITIVE
MOUSE 6	POSITIVE	POSITIVE
20     MOUSE 7	POSITIVE	POSITIVE
MOUSE 9	NEGATIVE	NEGATIVE
MOUSE 19	POSITIVE	POSITIVE
MOUSE 20	POSITIVE	POSITIVE
MOUSE 21	NEGATIVE	NEGATIVE
25     anti-FITC alone	ALL NEGATIVE	ALL NEGATIVE

**Liver**

5

	ENDOTHELIUM	BILE DUCT
129 MOUSE	POSITIVE	POSITIVE
MOUSE 6	POSITIVE	POSITIVE
MOUSE 7	POSITIVE	POSITIVE
MOUSE 9	NEGATIVE	NEGATIVE
MOUSE 19	POSITIVE	POSITIVE
MOUSE 20	POSITIVE	POSITIVE
MOUSE 21	NEGATIVE	NEGATIVE
anti-FITC alone	ALL NEGATIVE	ALL NEGATIVE

10

**Heart**

15

	ENDOTHELIUM	PERINUCLEAR	ENDO- MYOCARDIUM
129 MOUSE	POSITIVE	POSITIVE	POSITIVE
MOUSE 6	POSITIVE	POSITIVE	POSITIVE
MOUSE 7	POSITIVE	POSITIVE	POSITIVE
MOUSE 9	NEGATIVE	NEGATIVE	NEGATIVE
MOUSE 19	POSITIVE	POSITIVE	POSITIVE
MOUSE 20	POSITIVE	POSITIVE	POSITIVE
MOUSE 21	NEGATIVE	NEGATIVE	NEGATIVE
anti-FITC alone	ALL NEGATIVE	ALL NEGATIVE	ALL NEGATIVE

Lung

	ENDOTHELIUM	BRONCHI	PARENCHYMA	
129 MOUSE	POSITIVE	POSITIVE	POSITIVE	
MOUSE 6	POSITIVE	POSITIVE	POSITIVE	
MOUSE 7	POSITIVE	POSITIVE	POSITIVE	
5	MOUSE 9	NEGATIVE	NEGATIVE	NEGATIVE
MOUSE 19	POSITIVE	POSITIVE	POSITIVE	
MOUSE 20	POSITIVE	POSITIVE	POSITIVE	
MOUSE 21	NEGATIVE	NEGATIVE	NEGATIVE	
anti-FITC alone	ALL NEGATIVE	ALL NEGATIVE	ALL NEGATIVE	

10 These results indicate that IB<sub>4</sub> lectin does not bind  
kidney, heart, liver or lung tissue of the  $\alpha$ 1,3  
galactosyltransferase gene targeted (Gal KO) homozygous  
mice (mouse 21 and mouse 9). This confirms the lack of  
15 the galactose  $\alpha$ 1,3 galactose (GAL) epitope in the gene  
targeted (KO) mice. In contrast these antibodies react  
strongly with the tissues of a normal mouse and  
heterozygous KO mice (mouse 129, mouse 6, mouse 7, mouse  
19, mouse 20) of the same strain.

20 VI. RESISTANCE OF SPLEEN CELLS FROM KNOCKOUT MICE TO  
LYSIS BY HUMAN SERUM

Lysis of spleen cells by human serum was tested  
through use of a <sup>51</sup> chromium release assay. See in general  
Example 4, above.

25 A. Preparation of Mouse Splenocytes - Shortman,  
K.J. et al, Immunological Methods. 1:273-287 (1972).:  
-Dissect out spleen, avoid damaging outer membranes and  
carefully remove mesentery tissue and fat.  
-Place in petri dish, with 1 ml RPMI 1640 (Gibco BRL)  
/10% Heat-inactivated foetal calf serum (HI-FCS). (Heat-  
30 inactivation = 40 Min at 56°C).

-Gently tease out cells into petri dish, collect and centrifuge 500xg, 5 min, 4°C

-Remove RPMI/10% HIFCS, gently resuspend cells in 3 ml 0.9% NH4Cl (0.168M), using a Pasteur pipette. (Use 5 Pasteur pipettes or wide-bore pipettes for all re suspension and transfer procedures)

-Transfer to 10 ml tube, underlay with 1 ml HIFCS, stand on ice, 5 min.

-Transfer supernatant to clean tube, centrifuge 500xg, 7 10 min, 4°C

-Discard supernatant, re-suspend cells in 3 ml dead cell removal buffer, mix well with pipette.

-Pass through cotton wool plug in glass pipette (under gravity, do not force through), collect cells into 10 ml 15 tube.

-Underlay cells with 1 ml HI-FCS.

-Centrifuge 500xg, 7 min, 4°C

-Remove supernatant, re-suspend cells in 50  $\mu$ l RPMI, 10% HI-FCS. Store cells on ice.

20           B. Preparation of Serum:

Human -    Collect whole blood from a pool of normal donors; allow to stand at room temp. for 2 hours.

25           Wring the clot with an 'Orange stick'; spin Remove and pool serum. Store half at -70°C in 3 ml aliquot's (normal human serum); heat- inactivate the other half, see below.

Fetal calf serum - purchased from Gibco BRL, and stored at -20°C.

30           C. Cell Counting:

1.    Add 5  $\mu$ l cells to 95.0  $\mu$ l RPMI, 10% HI-FCS

2. Remove 10  $\mu$ l cells, add 10  $\mu$ l Acridine Orange/Et Br solution, (Lee, S.K. et al. Eur J. Immunol. 1975. 5: 259-262)

5 3. Count cells, (viable = green, deads = orange).  
4. Cell viability should be approx. 90-100 %  
5. Calculate cell number.

D.  $^{51}\text{Cr}$  Labelling:

	Cell Type	Incubation conditions	
		Time	Amount $^{51}\text{Cr}/10^7$ cells
10	Freshly prepared cells: (eg., splenocytes or lymphocytes)	~2 hours	~150-300 $\mu\text{Ci}$
15	Cultured Cells:	~1 hour	~100 $\mu\text{Ci}$
15	Labelling:		
20	Combine: - cells ( $2 \times 10^7$ ) - ( $^{51}\text{Cr}$ ) Sodium Chromate in 0.9% NaCl solution (the volume added depends on cell type as indicated above and on the specific activity of the - ( $^{51}\text{Cr}$ ) Sodium Chromate).	 - RPMI/2% HIFCS up to a total of 200 $\mu$ l	

Incubate at 37°C for time shown above with gentle agitation every 15 min.

25 E. Washing

-Place 4ml HI-FCS into 10ml tube and carefully layer labelling reaction on top with a swirling motion; centrifuge 5 min, 500xg, 4°C.  
-Remove top two layers with a careful circular motion using a glass pipette.  
-Resuspend cells in 1ml RPMI/2% HI FCS  
-Pellet cell suspension through another 4 ml HI FCS  
-Resuspend cell pellet in 1ml RPMI/2% HI FCS, store on ice.

F. Release Assay:

-Perform assay in 96-well microtire plate (ICN-FLOW).

-Assay should be set up in quadruplicate.

5 -Assay is performed in a total volume of 180  $\mu$ l.

Assay:

		NHS	*HI-NHS	16% SDS	CELLS	RPMI/2% HIFCS
10	MAX Release	-	90 $\mu$ l	22.5 $\mu$ l	25 $\mu$ l	42.5 $\mu$ l
	Spont. Release	-	90		25	65 $\mu$ l
	5% NHS	9 $\mu$ l	81		25	65
	10% NHS	18	72		25	65
	20% NHS	36	54		25	65
	30% NHS	54	36		25	65
15	40% NHS	72	18		25	65
	50% NHS	90	-		25	65

\*HI = heat inactivated

-All volumes indicated are in  $\mu$ l

-Reaction components are added to the plate in the order: RPMI, Serum and  $^{51}\text{Cr}$ -labelled cells.

20 -Cover plate with plate-sealer  
-Incubate, 4 hours, 37°C.  
-Spin plate, 1500 rpm, 5 min.  
-Remove plate-scaler, remove 80  $\mu$ l from each wall, count released chromium on gamma counter.  
25 -Calculate specific lysis for each well according to the formula:

$$\% \text{ Specific Lysis} = \frac{(\text{Test cpm} - \text{Spontaneous release cpm})}{100} \times \frac{(\text{Maximal release cpm} - \text{Spontaneous release cpm})}{}$$

30 Calculate mean and standard deviation for each experimental point. Graph % Human serum (X axis) against % Specific lysis (Y axis) for each type of cell (wild type, heterozygote KO and homozygous KO)

The results of these experiments are depicted in Figure 25. The results indicate that spleen cells from a homozygous knockout mouse are relatively resistant to lysis by human serum, in comparison to spleen cells derived from mice heterozygous for the interrupted allele or from wild-type mice.

EXAMPLE 16

Generation of Knockout Animals Through Microinjection of Eggs

Transgenic animals are generated routinely by microinjection of DNA into the pronuclei of fertilised 5 eggs. Generally this technology results in the random integration of the transgene in the genome. However, conventional transgenic technology has resulted in homologous recombination between the injected transgene and the endogenous gene. See, for example, Brinster et 10 al., Proc. Nat. Acad. Sci. USA 86: 1087-91 (1989).

Described below are procedures for inactivating the  $\alpha$ -1,3-Gal T gene in pigs through microinjection of eggs with gene targeting constructs.

I. GENE TARGETING CONSTRUCTS

15 The frequency of homologous recombination in embryos is improved if the gene targeting constructs are prepared with isogenic DNA. Therefore the "knock out" constructs are prepared from DNA isolated from the boar used to fertilize the oocytes used for microinjection. DNA is 20 isolated from the tail or ear tissue, and genomic fragments from both  $\alpha$ -1,3-Gal T alleles of the boar, encompassing exons 8 & 9 are cloned using long range PCR or conventional genomic library technologies. Clones for each of the  $\alpha$ -1,3-Gal T alleles are identified using 25 restriction fragment length polymorphism identification and DNA sequencing. Constructs to target both alleles are made by interrupting the coding sequence of exon 9, either by deletion or by inserting a heterologous DNA fragment. The constructs contain at least 8 kb of 30 homologous DNA to promote efficient homologous recombination.

Various approaches can be used to detect gene targeting events, depending on the strategies used in designing the knockout constructs. Several such

approaches, and the corresponding strategies for construction of constructs, are provided below:

5 a) PCR of Genomic DNA:

Homologous DNA on one side of the interrupting DNA fragment is constructed to be less than 1 kb, allowing PCR amplification of a short diagnostic fragment. (Amplification of small fragments generally is relatively efficient).

10 b) Reverse Transcription/PCR:

A deletion of about 100 bp within exon 9 is made, allowing synthesis of a shortened  $\alpha$ -1,3-Gal T mRNA in correctly targeted cells. The shortened mRNA is detected by RT/PCR, using primers that amplify a fragment extending from exon 8 and encompassing the deletion site.

15 c) Green Fluorescent Protein (GFP) gene expression:

20 GFP is a protein from the bioluminescent jelly fish *Aequorea victoria*. It absorbs blue light (395 nm) and fluoresces to emit green light (509 nm). GFP is a useful marker for gene expression. Chafie et al., Green Fluorescent Protein as a Marker for Gene Expression.

25 Science 263: 802-5 (1994). The  $\alpha$ -1,3-Gal T gene is interrupted within exon 9 by in-frame insertion of the GFP coding region. Expression of the GFP gene (with resulting fluorescence at 509 nm) is driven by the  $\alpha$ -1,3-Gal T gene promoter in correctly targeted cells.

30 II. GENERATING EMBRYOS FOR MICROINJECTION

Fertilized embryos are generated as described by Nottle et al., (1993). Proc Aust Soc for Reproductive Biol 26, 33. The protocol involves:

5           a) Sperm from the boar providing DNA for the targeting construct is collected and stored frozen in liquid N<sub>2</sub>.

10          b) Superovulation of donor gilts:  
              Gilts are mated at the second oestrus, and aborted between days 25-40 days of gestation to synchronise the subsequent oestrus cycles. Abortion is achieved by intramuscular injection of 1 mg cloprostenol (a prostaglandin F2 $\alpha$  analogue), followed by a second 0.5 mg injection 24 hours later. Gilts are superovulated by injection of 1000 i.u. equine chorionic gonadotrophin (eCG) or pregnant mare serum gonadotrophin at the time of the second cloprostenol injection, and a subsequent injection 72 hours later of 500 i.u. human chorionic gonadotrophin (hCG).

15          c) Fertilization:  
              Superovulated gilts are artificially inseminated 20-30 hours after the hCG injection, followed by a second insemination 2-4 hours later, with semen from the boar that provided DNA for the targeting construct.

20          d) Embryo collection:  
              Embryos are collected surgically 50-56 hours after hCG injection prior to fusion of the pronuclei. Oviducts are flushed with 15-20 ml phosphate saline buffer containing 1% fetal calf serum. One-cell embryos are recovered by searching oviductal flushings using low magnification microscopy.

25

30

### III. MICROINJECTION OF EMBRYOS

Embryos are centrifuged at 12000 x g for 8 min to stratify the cytoplasm and allow the pronuclei to

be visualised, and held in Dulbecco's Minimal Essential Medium with 25 mM Hepes and 5 mg/ml bovine serum albumin. Pronuclei are injected, using differential interference contrast optics, with 4-10 picolitres of DNA (10 ng/ $\mu$ l) in PBS. Gene targeting with isogenic DNA is maximized by coinjecting both allelic constructs derived from the boar into the male pronucleus.

IV. TRANSFER OF INJECTED EMBRYOS TO RECIPIENT GILTS

The oestrus cycles of recipient gilts are synchronized with those of donors. The recipients are mated and aborted using the protocol described above, and injected with 500 i.u. eCG. Injected embryos are transferred surgically (20-40 per oviduct) to recipients on the same day that they are collected from donor gilts.

V. SCREENING FOR HOMOLOGOUS RECOMBINATION

Homologous recombinants can be detected by analysis of tissue from the born piglets. Screening procedures involve PCR technology, the precise strategy depending on the design of the gene targeting construct. Because many  $\alpha$ -1,3-Gal T mRNA molecules are synthesized from a single  $\alpha$ -1,3-Gal T gene in expressing cells, the RT/PCR approach can be more sensitive than PCR amplification of genomic DNA. The RT/PCR screening strategy relies on successful transcription of the interrupted gene and relative stability of the shortened mRNA.

Alternatively, constructs that promote expression of heterologous genes (eg: GFP) in correctly targeted cells allow embryos to be screened at the blastocyst stage for marker gene expression (i.e.: GFP expression can be detected by measuring fluorescence within blastocysts at 509 nm). The microinjected embryos are cultured *in vitro*

until blastocyst development, screened for fluorescence, and fluorescing embryos transferred into recipients.

EXAMPLE 17

A Novel Variant of Leukemia Inhibitory Factor (LIF)

5 Previous reports have demonstrated the existence of two forms of murine LIF. The original form (from the D transcript) was expressed and commercialized by AMRAD Corporation Ltd (Kew Victoria, Australia). The protein product derived from this transcript (hereinafter "D-LIF") is sold commercially by AMRAD as "ESGRO". Another 10 form of LIF (hereinafter "M-LIF"), derived from an alternative transcript, is described in US Patent Application No. 07/994,099 and in Rathjen et al., Cell 62: 1105-14 (1990). The present inventors have now found 15 a third transcript of LIF (hereinafter "T-LIF") which is found in ES cells and in human teratocarcinoma-derived cell lines such as the GCT 27 teratocarcinoma-derived cell lines described by Pera et al., Differentiation 42: 10 (1989).

20 The T-LIF protein is found intracellularly in contrast to the other two forms of LIF which are both extracellular. The transcript was cloned using the RACE PCR technique (see below) from murine ES cells and human GCT 27 teratocarcinoma-derived cell lines, and sequenced 25 using standard methods. The presence of the T-LIF transcript was confirmed by PCR analysis of ES cell mRNA and RNA'ase protection on GCT 27 RNA. The transcript comprises a novel first exon, located in the first intron of the LIF gene, spliced to the known exon 2 and exon 3 30 sequences. The mouse nucleotide sequence (SEQ ID NO: 25) and deduced amino acid sequence (SEQ ID NO: 26) are set out in Figure 26. The human nucleotide sequence (SEQ ID NO: 31) and deduced amino acid sequence (SEQ ID NO: 32) are set out in Figure 27.

When expressed in a COS cell expression system, the murine T-LIF transcript produces a 17 kD protein that is unglycosylated (D-LIF is glycosylated in the Golgi during the secretion process) (Figure 28). Translation of T-LIF initiates at the first in-frame initiation codon (ATG) in exon 2 to produce a protein of 158 amino acids. The protein is 45 amino acids shorter than the unprocessed D-LIF protein and 22 amino acids shorter than the mature D-LIF product generated by cleavage of the signal sequence. Because the T-LIF protein does not contain a signal sequence, it does not leave the cell and is unglycosylated. The T form of LIF is efficacious in preventing the differentiation of ES cells in culture.

METHODS

15 RACE cDNA CLONING

Cytoplasmic RNA (10 $\mu$ g) from CP1 murine ES cells (Bradley et al., Nature 309: 255-56 (1984) was reverse transcribed from the oligonucleotide 5'ACACGGTACTTGTTGCA-3' (SEQ ID NO: 27), which hybridizes to residues 500-484 of the murine LIF cDNA. The RNA was added to 20 pmol of primer and 2 $\mu$ l of 10x annealing buffer (500mM Tris-HCl (pH 8.0), 60mM MgCl<sub>2</sub>, 400mM KCl) in a total volume of 16 $\mu$ l, heated to 85°C for 5 min, and cooled slowly to room temperature. The elongation reaction was carried out as described by Frohman et al. (Proc. Natl. Acad. Sci. USA 85: 8998-9002 (1988)). Excess oligonucleotide was removed by gel filtration through a 2ml Sephadryl S-400 (Pharmacia) column equilibrated with 0.05 x TE (TE = 10mM Tris-HCl pH 7.6, 1.0mM EDTA). Fractions of 50 $\mu$ l corresponding to the cDNA radioactive peak were pooled, concentrated by vacuum centrifugation, and resuspended in 23 $\mu$ l of H<sub>2</sub>O. To tail the 3'-end of the cDNA with dG residues, 3 $\mu$ l of 10mM dGTP and 6 $\mu$ l of 5 x tailing buffer (Bethesda Research Laboratories) were added and the

mixture was incubated at 37°C for 60 min. and then at 70°C for 15 min. After ethanol precipitation, the cDNA template was resuspended in 500μl H<sub>2</sub>O.

PCR was carried out using a mouse LIF specific 5 oligonucleotide, 5'-TTCTGGTCCCAGGGTGATATTGGTCA-3' (residues 389-365) (SEQ ID NO: 28), and an anchor oligonucleotide, 5'-CCATGGCCTCGAGGGCCCCCCCCCCCC-3' (SEQ ID NO: 29). PCR was carried out in a final volume of 50μl containing 7μl of the cDNA template and 34pmol of 10 each oligonucleotide. Reaction conditions were as recommended by Perkin-Elmer Cetus, with a final concentration of 1.5mM MgCl<sub>2</sub>. DNA was denatured prior to the addition of Taq polymerase (Perkin-Elmer Cetus) by heating the reaction mixture to 94°C for 5 min. Each PCR 15 cycle (35 in total) consisted of denaturation for 2 min at 94°C, annealing for 2 min at 55°C, and elongation for 3 min at 72°C. After the final elongation (30 min at 72°C), samples were ethanol precipitated, digested with SmaI and XhoI and analyzed by agarose gel 20 electrophoresis. DNA was purified from agarose gels using Geneclean and cloned into SalI- and SmaI- digested TST7 19U (Stratagene). Suitable recombinant plasmids were purified by the rapid boiling method.

Double-stranded sequencing was performed with 25 Sequenase version 2.0 (USB) according to the manufacturers recommendations.

#### BIOLOGICAL ASSAY FOR LIF ACTIVITY

An undifferentiated, murine ES cell culture (MBL5; Pease et al., Dev. Biol. 141: 344-52 (1990), between 30 passages 15 and 30) is trypsinized and made into a single cell suspension. The cells are pelleted by centrifugation and resuspended in complete ES Cell Medium without LIF (DMEM (without Hepes), 10% FCS, 1mM βME, 1mM glutamine). The cells are then seeded into 24-well

microtitre plates at  $5 \times 10^2$  cells/16 mm well containing 1 ml of ES Cell Medium without LIF.

The complete T-LIF open reading frame was reconstructed from the PCR product and inserted into the 5 COS cell expression vector pXMT2 as described by Rathjen et al., Cell 62: 1105-14 (1990). The plasmid used for transfection of COS cells is shown in Figure 29. The COS cells were transfected by electroporation. Supernatants from COS cells expressing T-LIF were added to the above 10 ES cells in various dilutions (1/5, 1/10, 1/50, 1/100, 1/50, 1/1000) and incubated for 4 days in an incubator with 10% CO<sub>2</sub>. Controls used supernatants from COS cells expressing D-LIF (pDR1, Rathjen et al., Cell 62: 1105-14 (1990)).

15 LIF activity is assessed as present if cells morphologically resemble ES-cells after 4 days and are distinct from the controls incubated without any form of LIF. The ES-cells are also stained for alkaline phosphatase as undifferentiated ES-cells are positive for 20 this marker.

Even though T-LIF is produced intracellularly, sufficient numbers of cells lyse to give significant amounts of LIF activity in the culture supernatants. If the COS cells expressing T-LIF are lysed, more LIF 25 activity is released.

#### PCR DETECTION OF T-LIF TRANSCRIPT

PCR was carried out on ES cell cDNA (prepared as described above except that the cDNA was not tailed with dG). PCR conditions were as described above except that 30 2mM MgCl<sub>2</sub> was used in the reactions. The oligonucleotides 5'-CACCTTCGCTTCCT-3' (SEQ. ID NO. 30) and 5'-TTCTGGTCCC GG GTGATATTGGTCA-3' (SEQ. ID. NO 28) were used at 80 picograms/reaction. Products of the PCR reaction were ethanol precipitated as described above,

separated electrophoretically on a 2% agarose gel and transferred to a nylon membrane for detection using Southern hybridization (Figure 30). The probe was the full length D-LIF transcript isolated from pDR1 (Rathjen 5 et al., Cell 62: 1105-14 (1990)). The control experiment is designed to detect all LIF transcripts using internal primers 5'-TTCTGGTCCCGGGTGATATTGGTCA-3' (SEQ. ID. NO 28) and 5' -CTGTTGGTTCTGCACTGGA-3' (SEQ. ID. NO. 33).

The foregoing detailed description has been provided 10 for a better understanding of the invention only and no unnecessary limitation should be understood therefrom as some modifications will be apparent to those skilled in the art without deviating from the spirit and scope of the appended claims.

15           What is claimed is:

1       1. A purified and isolated nucleic acid molecule  
2 comprising a nucleic acid sequence selected from the  
3 group consisting of (1) the porcine nucleic acid sequence  
4 depicted in Figure 4 (SEQ ID NO: 7), (2) a sequence  
5 corresponding to the sequence of (1) within the scope of  
6 the degeneracy of the genetic code, (3) a sequence that  
7 encodes a porcine polypeptide having  $\alpha$ -1,3  
8 galactosyltransferase activity and that hybridizes under  
9 standard high stringency conditions with a sequence  
10 complementary to the sequence of (1) or (2), and (4) a  
11 sequence complementary to the sequence of (1), (2) or  
12 (3).

1       2. A host cell that is transformed with the  
2 nucleic acid molecule of claim 1.

1       3. A porcine  $\alpha$ -1,3 galactosyltransferase encoded  
2 by the nucleic acid molecule of claim 2.

1       4. A DNA construct useful for inactivating the  
2 porcine  $\alpha$ -1,3 galactosyltransferase gene by insertion of  
3 a desired DNA sequence into an insertion site of said  
4 gene, comprising said desired DNA sequence flanked by  
5 first and second homology sequences, said first and  
6 second homology sequences being, respectively,  
7 sufficiently homologous to first and second genomic  
8 sequences flanking said insertion site to allow for  
9 homologous recombination of said DNA construct with said  
10 porcine  $\alpha$ -1,3 galactosyltransferase gene when said DNA  
11 construct is introduced into a porcine cell having said  
12  $\alpha$ -1,3 galactosyltransferase gene.

1       5. The DNA construct of claim 4, wherein said  
2 insertion site is within exon 4, exon 7, exon 8 or exon 9  
3 of the porcine  $\alpha$ -1,3 galactosyltransferase gene.

1       6. The DNA construct of claim 4, wherein said  
2 desired DNA sequence is selected from the group  
3 consisting of the neo<sup>R</sup> gene, the hyg<sup>R</sup> gene and the  
4 thymidine kinase gene.

1       7. The DNA construct of claim 6, wherein said  
2 desired DNA sequence is bordered at the 5' and 3' ends by  
3 FRT DNA elements, and wherein stop codons for each of the  
4 three reading frames have been inserted 3' to the desired  
5 DNA sequence.

1       8. A DNA construct useful for inactivating the  
2 murine  $\alpha$ -1,3 galactosyltransferase gene by insertion of a  
3 desired DNA sequence into an insertion site of said gene,  
4 comprising said desired DNA sequence flanked by first and  
5 second homology sequences, said first and second homology  
6 sequences being, respectively, sufficiently homologous to  
7 first and second genomic sequences flanking said  
8 insertion site to allow for homologous recombination of  
9 said DNA construct with said murine  $\alpha$ -1,3  
10 galactosyltransferase gene when said DNA construct is  
11 introduced into a murine cell having said  $\alpha$ -1,3  
12 galactosyltransferase gene.

1       9. The DNA construct of claim 8, wherein said  
2 insertion site is within exon 4, exon 7, exon 8 or exon 9  
3 of the murine  $\alpha$ -1,3 galactosyltransferase gene.

1        10. The DNA construct of claim 8, wherein said  
2        desired DNA sequence is selected from the group  
3        consisting of the neo<sup>R</sup> gene, the hyg<sup>R</sup> gene and the  
4        thymidine kinase gene.

1        11. The DNA construct of claim 10, wherein said  
2        desired DNA sequence is bordered at the 5' and 3' ends by  
3        FRT DNA elements, and wherein stop codons for each of the  
4        three reading frames have been inserted 3' to the desired  
5        DNA sequence.

1        12. A method for generating a mammalian totipotent  
2        cell having at least one inactivated  $\alpha$ -1,3  
3        galactosyltransferase allele, said totipotent cell  
4        derived from a mammalian species having a functional  $\alpha$ -  
5        1,3 galactosyltransferase gene, comprising:  
6                (a) providing a plurality of cells characterized as  
7        totipotent cells of said mammalian species;  
8                (b) introducing into said totipotent cells a nucleic  
9        acid construct effective for inactivating said  $\alpha$ -1,3  
10        galactosyltransferase gene by insertion of a desired DNA  
11        sequence into an insertion site of said gene through  
12        homologous recombination; and  
13                (c) identifying a totipotent cell having at least  
14        one inactivated  $\alpha$ -1,3 galactosyltransferase allele.

1        13. The method of claim 12 in which said totipotent  
2        cell is a murine ES cell.

1        14. The method of claim 12 in which said totipotent  
2        cell is a murine egg.

1        15. The method of claim 12 in which said totipotent  
2        cell is a porcine ES cell.

1        16. The method of claim 12 in which said totipotent  
2 cell is a porcine PGC.

1        17. The method of claim 12 in which said totipotent  
2 cell is a porcine egg.

1        18. A method for generating a mammal lacking a  
2 functional  $\alpha$ -1,3 galactosyltransferase gene, said mammal  
3 belonging to a species having a functional  $\alpha$ -1,3  
4 galactosyltransferase gene, comprising:

5            (a) providing a mammalian totipotent cell having at  
6 least one inactivated  $\alpha$ -1,3 galactosyltransferase allele,  
7 said totipotent cell derived from a mammalian species  
8 having a functional  $\alpha$ -1,3 galactosyltransferase gene;

9            (b) manipulating said totipotent cell such that  
10 mitotic descendants of said cell constitute all or part  
11 of a developing embryo;

12            (c) recovering a neonate derived from said embryo;  
13 and

14            (d) raising and breeding said neonate to obtain a  
15 mammal homozygous for said inactivated  $\alpha$ -1,3  
16 galactosyltransferase allele.

1        19. The method of claim 18, wherein said totipotent  
2 cell is a murine ES cell and said manipulating comprises  
3 injecting said ES cell into the blastocyst cavity of a  
4 murine blastocyst and implanting said injected blastocyst  
5 into a murine recipient female.

1        20. The method of claim 18, wherein said totipotent  
2 cell is a murine egg, and said manipulating comprises  
3 implanting said egg into a murine recipient female.

1        21. The method of claim 18, wherein said totipotent  
2 cell is a porcine ES cell and said manipulating comprises  
3 injecting said ES cell into the blastocyst cavity of a  
4 porcine blastocyst and implanting said injected  
5 blastocyst into a porcine recipient female.

1        22. The method of claim 18, wherein said totipotent  
2 cell is a porcine ES cell and said manipulating comprises  
3 injecting said ES cell into a porcine morula.

1        23. The method of claim 18, wherein said totipotent  
2 cell is a porcine ES cell and said manipulating comprises  
3 co-culture of said ES cell with a zona pellucida-  
4 disrupted porcine morula.

1        24. The method of claim 18, wherein said totipotent  
2 cell is a porcine ES cell and said manipulating comprises  
3 fusing said ES cell with an enucleated porcine zygote.

1        25. The method of claim 18, wherein said totipotent  
2 cell is a porcine egg, and said manipulating comprises  
3 implanting said egg into a porcine recipient female.

1        26. A mammal lacking a functional  $\alpha$ -1,3  
2 galactosyltransferase gene, said mammal belonging to a  
3 species having a functional  $\alpha$ -1,3 galactosyltransferase  
4 gene, said mammal produced by the method of claim 18.

1        27. The mammal of claim 26, wherein said mammal is  
2 a mouse.

1        28. The mammal of claim 26, wherein said mammal is  
2 a pig.

1        29. A non-naturally occurring mammal lacking a  
2        functional  $\alpha$ -1,3 galactosyltransferase gene, said mammal  
3        belonging to a species having a functional  $\alpha$ -1,3  
4        galactosyltransferase gene.

1        30. The mammal of claim 29, wherein said mammal is  
2        a mouse.

1        31. The mammal of claim 29, wherein said mammal is  
2        a pig.

1        32. A purified and isolated nucleic acid molecule  
2        comprising a nucleic acid sequence selected from the  
3        group consisting of (1) the nucleic acid sequence  
4        depicted in Figure 26 (SEQ ID NO: 25), (2) a sequence  
5        corresponding to the sequence of (1) within the scope of  
6        the degeneracy of the genetic code, (3) a sequence that  
7        encodes murine T-LIF and that hybridizes under standard  
8        high stringency conditions with a sequence complementary  
9        to the sequence of (1) or (2), and (4) a sequence  
10      complementary to the sequence of (1), (2) or (3).

1        33. A host cell that is transformed with the  
2        nucleic acid molecule of claim 32.

1        34. A murine T-LIF polypeptide encoded by the  
2        nucleic acid molecule of claim 32.

1       35. A purified and isolated nucleic acid molecule  
2 comprising a nucleic acid sequence selected from the  
3 group consisting of (1) the nucleic acid sequence  
4 depicted in Figure 27 (SEQ ID NO: 31), (2) a sequence  
5 corresponding to the sequence of (1) within the scope of  
6 the degeneracy of the genetic code, (3) a sequence that  
7 encodes human T-LIF and that hybridizes under standard  
8 high stringency conditions with a sequence complementary  
9 to the sequence of (1) or (2), and (4) a sequence  
10 complementary to the sequence of (1), (2) or (3).

1       36. A host cell that is transformed with the  
2 nucleic acid molecule of claim 35.

1       37. A human T-LIF polypeptide encoded by the  
2 nucleic acid molecule of claim 35.

1       38. A method for eliminating or reducing hyperacute  
2 rejection of non-primate mammalian cells, tissues and  
3 organs by human serum, comprising adding, to said human  
4 serum, a physiologically acceptable amount of galactose  
5 or a saccharide in which the terminal carbohydrate is an  
6  $\alpha$  galactose linked at position 1, prior to exposure of  
7 said human serum to said non-primate cells, wherein said  
8 amount of galactose or saccharide is sufficient to reduce  
9 or eliminate said hyperacute rejection.

1       39. The method of claim 38, wherein said saccharide  
2 is selected from the group consisting of melibiose,  
3 galactose  $\alpha$ 1-3 galactose and stachyose.

1       40. A method for eliminating or reducing hyperacute  
2 rejection of non-primate mammalian cells, tissues and  
3 organs by human serum, comprising substantially depleting  
4 said serum of immunoglobulin.

1        41. A method for eliminating or reducing hyperacute  
2 rejection of non-primate mammalian cells, tissues and  
3 organs by human serum, comprising substantially depleting  
4 said serum of IgM antibodies.

1        42. A method for eliminating or reducing hyperacute  
2 rejection of non-primate mammalian cells by human serum,  
3 comprising substantially depleting said serum of anti-GAL  
4 IgM and IgG antibodies.

1        43. A method for eliminating or reducing hyperacute  
2 rejection of non-primate mammalian cells by human serum,  
3 comprising substantially depleting said serum of anti-GAL  
4 IgM antibodies.

1        44. Affinity-treated human serum substantially free  
2 of anti-GAL antibodies.

1        45. Affinity-treated human serum substantially free  
2 of anti-GAL IgM antibodies.

ABSTRACT OF THE DISCLOSURE

Human pre-formed xenoantibodies play an important role in the hyperacute rejection response in human xenotransplantation. Disclosed are materials and methods for removing or neutralizing such antibodies. Also disclosed are materials and methods for reducing or eliminating the epitopes in the donor organs that are recognized by such antibodies. Such epitopes are formed as the result of activity by the enzyme  $\alpha$ -1,3 galactosyltransferase. The porcine gene encoding  $\alpha$ -1,3 galactosyltransferase is disclosed, as are materials and methods for inactivating ("knocking out") the  $\alpha$ -1,3 galactosyltransferase gene in mammalian cells and embryos. Included are nucleic acid constructs useful for inactivating the  $\alpha$ -1,3 galactosyltransferase gene in a target cell. Also disclosed is a novel leukemia inhibitory factor (T-LIF) that is useful for maintenance of embryonic stem cells and primordial germ cells in culture.

20 110464.B11

Fig. 1

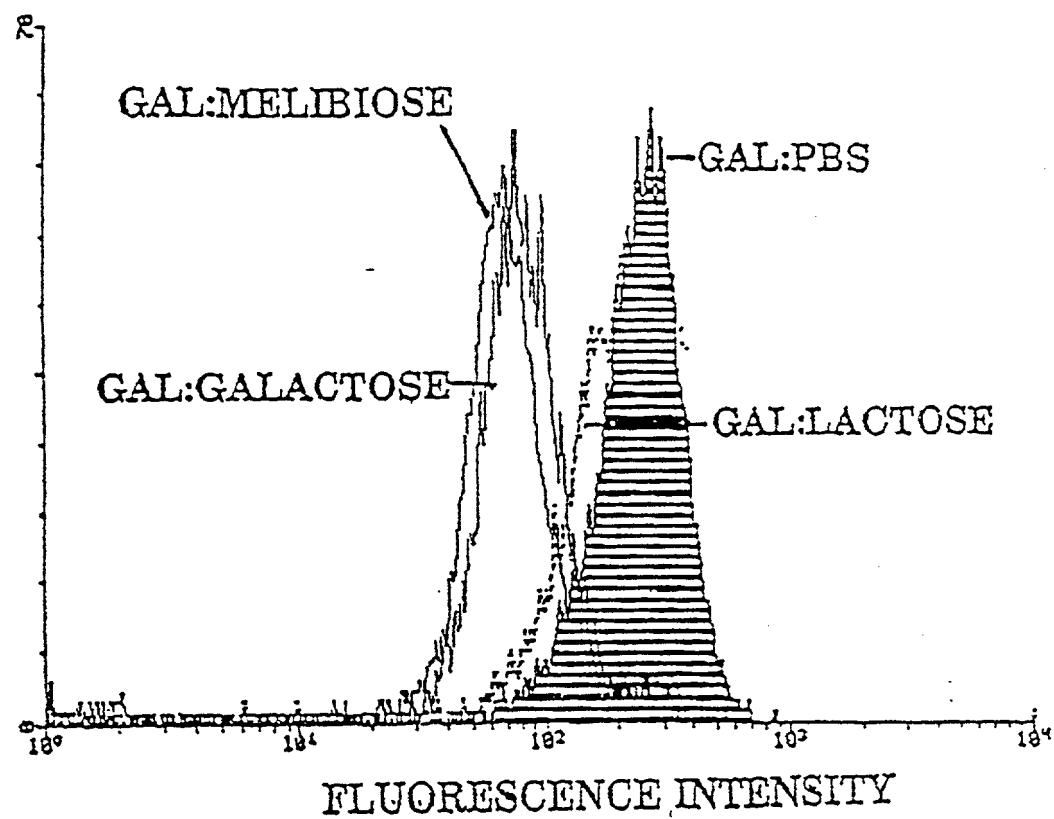
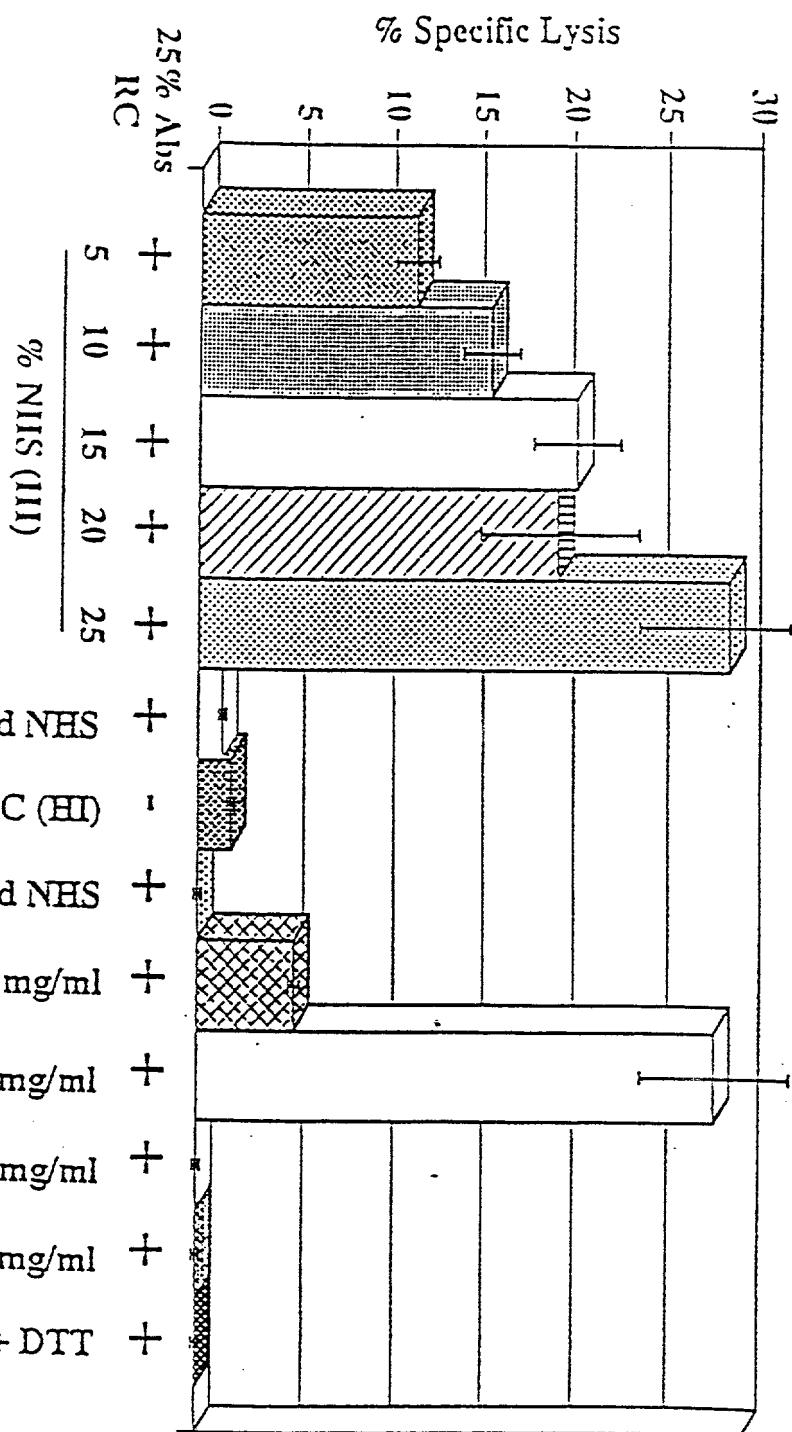


FIGURE 2



(a)

(b)

(c)

(d)

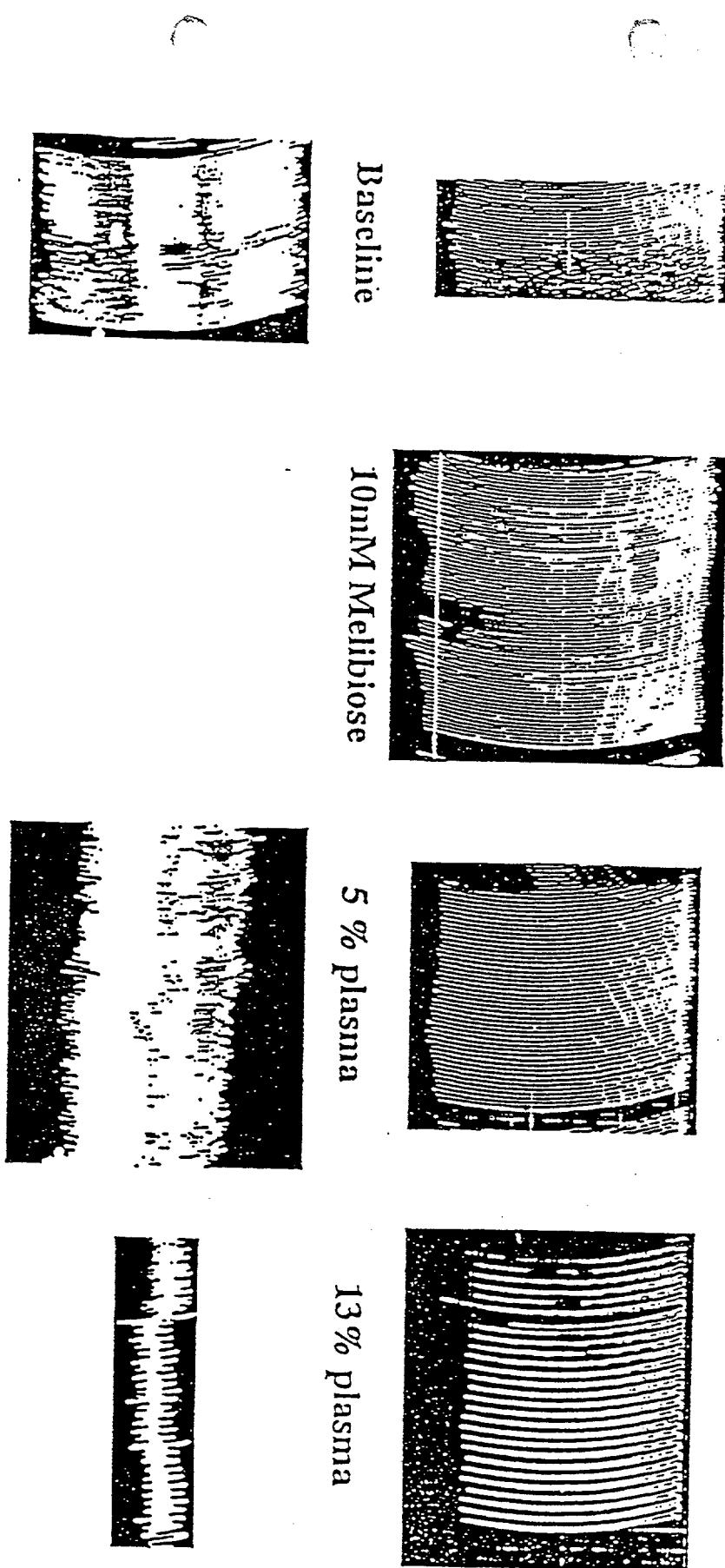
(e)

(f)

(g)

(h)

FIGURE 3



F<sub>1</sub>  
PBM line-up (nt):

PCTCD	1	-----	-----	-----	50		
BOVGSTA	1	CCGGCGGCCG	CCCCGAGCTG	CCAGCTCTGA	CCCCCTGCC	CCCCCCCC	50
MUSCLYTNS	1	-----	-----	-----	-----	-----	50
PCTCD	51	-----	-----	-----	100		
BOVGSTA	51	CCGCTCCCTG	CCCCCTCTGC	CCCCCCCC	CCAGCTGCC	CCCCCCCC	100
MUSCLYTNS	51	-----	-----	-----	-----	-----	100
PCTCD	101	-----	-----	-----	150		
BOVGSTA	101	CCCCAAGCGA	CCCCACCCCG	ACACCCCCCG	CCCCACCC	CCCCCCCC	150
MUSCLYTNS	101	-----	-----	-----	-----	-----	150
PCTCD	151	-----	-----	-----	200		
BOVGSTA	151	CCAGGAAACCG	ACCCCCCCCC	CTTCTTCCCC	ACCCCCACAC	CCCCCCCC	200
MUSCLYTNS	151	-----	-----	-----	-----	-----	200
PCTCD	201	-----	-----	-----	250		
BOVGSTA	201	ACCCCAAGCG	CCGGCCACCG	CCCCACCCCG	CCAGCTCTGA	CCCCACCC	250
MUSCLYTNS	201	-----CG	TCTTACGGAG	CTGAGATTC	TGGGTGGAGC	CTTACCCCTG	250
PCTCD	251	-----	-----	-----	300		
BOVGSTA	251	AAACCCACCTG	CTCTCAAGTG	CCACACCCAGC	TCTCCCGAAAG	ACGAACTTGC	300
MUSCLYTNS	251	CTTTTTCTTA	CTGGGCTGAC	ACCTTCCCTT	CTAGACTCTT	CTTGGAAATGA	300
Exon 1 ↓ Exon 2							
PCTCD	301	-----	-----	-----	350		
BOVGSTA	301	CTGGCATTTG	CACTGAAAGA	CCAGACACTT	CACTAAATCA	ACGAACTGAG	350
MUSCLYTNS	301	GAAGTACCGA	TTCCTGAGA	GAACCTGGGG	TCTCAGGTC	TGGAGTTGG	350
PCTCD	351	-----AGC	CTTCCCTCT	TCTCCAGACG	400		
BOVGSTA	351	AAAGGCTGGAC	CTTCCCTCTC	TCCC---AGC	CTTCCCTCT	TCTCCAGAC	400
MUSCLYTNS	351	AAACCTGTAC	CTTCCCTTCC	TCTGCTGAGC	CTTCCCTCT	TCCCAGCCC	400
Exon 2 ↓ Exon 3							
PCTCD	401	AGACCTCACT	AGAACTT-GT	TTC---GCC	TTTACTCTG	GGGGAGACAG	450
BOVGSTA	401	GGAGCTCACT	AGAACTT-GG	TACTTTGCC	TTTACTCTA	GGGGAGACAG	450
MUSCLYTNS	401	AGACCTGGAC	AGACCTCCG	TTCGTTTCT	CTTGGCTTTC	GGGGAGACAC	450
Exon 2 ↓ Exon 3							
PCTCD	451	AGCAGAGGAT	CAG-----	-----	500		
BOVGSTA	451	ACGAGACCAT	CAG-----	-----	500		
MUSCLYTNS	451	ACCTCACCAT	CAACCTCACT	TTCACACTAA	CAACCTGAGTC	500	
PCTCD	501	-----	-----	-----	550		
BOVGSTA	501	-----	-----	-----	550		
MUSCLYTNS	501	TCCTGGATT	AAACCCCTCT	ACTACCTTCC	CTCCACCTAA	CACTTTCTAC	550
PCTCD	551	-----	-----	-----	600		
BOVGSTA	551	-----	-----	-----	600		
MUSCLYTNS	551	ATCACTATAC	TTCAGAATCT	CCAGCTGAC	AAAGATCTCCA	TCTCAAGTC	600

Fig 4 (cont) 4-2

Fig 4 (cont) 4-3

PCTCD	1251	AAACACATCAG CATGATGCCG ATGAAACACCA TCGGGGACCA CATCCTGCCG	1300
BOVGSTA	1251	ACGACATCAG CATGATGCCG ATGAAACACCA TCGGGGACCA CATCCTGCCG	1300
MUSGLYTNS	1251	ACGATATCAG CATGATGCCG ATGAAACACCA TTGGGGACCA CATCCTGCCG	1300
PCTCD	1301	CACATCCAGC ACCACCTGGA CTTCCTCTTC TCCATCCACG TCCATCAAGT	1350
BOVGSTA	1301	CACATCCAGC ATGACCTTCG CTTCCTCTTC TCCATCCACG TCCACCAAGT	1350
MUSGLYTNS	1301	CACATCCAGC ACCACCTGCA CTTCCTCTTC TCCATCCACG TCCATCAAGT	1350
PCTCD	1351	CTTCCAAAC AACCTTGGGG TCCACACCCCT GGGGAGTCC CTGGCTCAC	1400
BOVGSTA	1351	CTTCCAAAGAC AACCTTGGGG TCCACACCCCT GGGGAGTCC CTGGCCAC	1400
MUSGLYTNS	1351	CTTCCAAAGAC AACCTTGGGG TCCACACCCCT GGGGAGTCC CTGGCAC	1400
PCTCD	1401	TACAGGCCTG CTGGTACAAAG CCACATCTCA ACAGCTTCAC CTACCAAC	1450
BOVGSTA	1401	TACAGGCCTG CTGGTACAAAG CCACATCTCA ACAGCTTCAC CTACCAAC	1450
MUSGLYTNS	1401	TCCAGGCCTG CTGGTACAAAG CCACATCTCA ACAGCTTCAC CTACCAAC	1450
PCTCD	1451	CCCAACCACT CCCACACCCCTA CATTCCCTTT GGGGAGGGGG ATTTTTTATTA	1500
BOVGSTA	1451	CCCAACCACT CTCCACACATA CATTCCCTTC GGGGAGGGGG ATTTTTTATTA	1500
MUSGLYTNS	1451	CCGGAACTGT CGGGCCCTTA CATTCCATTC GGGGAGGGGG ATTTTTTATTA	1500
PCTCD	1501	CCACGGCACCC ATTTTTGGGG GAAACACCCAC TCAAGCTCTA AACATCACTC	1550
BOVGSTA	1501	CCATGGAGCC ATTTTTGGGG GAAACACCCAC TCAAGCTCTT AACATCACCC	1550
MUSGLYTNS	1501	CCACGGGGCC ATTTTTGGAG GAAACACCTAC TCAACATTCTC AACCTCACCA	1550
PCTCD	1551	AGGACTCTT CAAAGGAATC CTCCAGGACA ACAGAAATCA CATAGAAGCC	1600
BOVGSTA	1551	ACCAATCTT CAAAGGAATC CTCCAGGACA ACAGAAATCA CATAGAAGCC	1600
MUSGLYTNS	1551	GGGACTCTT TAAGGGCTAC CTCCAGGACA ACAGAAATCA CATAGAAGCC	1600
PCTCD	1601	CACTGGCATC ATCAAAACCA TCTAAACAAAG TAACTTAAATTG TCAACAAAC	1650
BOVGSTA	1601	CAATGGCATC ATCAAAACCA TCTAAACAAAG TAACTTCTTC TCAACAAAC	1650
MUSGLYTNS	1601	CACTGGCATC ATCACACCCAA CCTCACACAA TAACTTCTTC TCAACAAAC	1650
PCTCD	1651	CACTAAATC TTATCCCCAG AATACTCTG GGATTATCAT ATACCCATCT	1700
BOVGSTA	1651	TACTAAATC TTATCCCCGG AATACTCTG GGATTATCAT ATACGGCTAC	1700
MUSGLYTNS	1651	CACTAAATC CTATCTCCAG ACTATCTG GGACTATCAT ATACCCCTCC	1700
PCTCD	1701	CTCTCCATAT TACCATCTG AAGATACCTT CCCACACAAA ACACATATAAT	1750
BOVGSTA	1701	CTCGGGATAT TAAGCTCTG AAGATCTCTT CCCACACAAA ACACATATAAT	1750
MUSGLYTNS	1701	CTTCAGATAT TAAAGCTCTG AAGCTACCTT CCCACACAAA ACACATATAAT	1750
Stop			
PCTCD	1751	TTGGTTAGAA ATAAATCTG ACTTAAATT CTGGCACCAAG TTTTCTGAAAT	1800
BOVGSTA	1751	CTGGTTAGAA ATAAATCTG ACTT-----T CTGGCACCAAG ATTTCTGAAAT	1800
MUSGLYTNS	1751	TTGGTTAGAA ATAAATCTG ACTTCAAAATT GTG----- --ATGGAAAC	1800
PCTCD	1801	TTCAAAACAGT ATTAATCTGG CTACTTCTTC ACACAACTAC ---CACTTA	1850
BOVGSTA	1801	TTCAACAGCT ATTAATCTGG CTACTTCTTC ACACAACTAA ---CACTTA	1850
MUSGLYTNS	1801	TTCAACAC--T ATTAATCTGG CTACTTCTTC AAACAACTAC CAACACTTCA	1850

Fig. 4 (cont) 4 - 4

PCTCD	1851	TTTTAAGTTT TAAAGGATAA CTAAAGAA- ---TACCA CACAGTAA-G	1900
BOVGSTA	1851	TTTTAAGTTA AAAAAAGATA CTAAAGAA- ---TACCA CACAGTAA-G	1900
MUSGLYTNs	1851	TTTCAACTTT TAAAGAGAA CTAAAGAAAC TACCAAGCA	1900
PCTCD	1901	TACATATTAT TCTTCCTTGC AACCTTCAGG CTTCATTAAT CCCACAAATCA	1950
BOVGSTA	1901	TACATATTAT TCTTCCTTGT AACCTTCAGG CTTCATTAATAC CCCACAAATCA	1950
MUSGLYTNs	1901	AACACATCAT TCTTCCT-GA CACCTTCAGG CT-CTATAT CTCAAGAAAC	1950
PCTCD	1951	CTCTGTGG-- --TAATCAAA TCTAAATTCC CACTGATTC .....	2000
BOVGSTA	1951	ACCTGTGG-- --TAATCAAA TCTAAATTCC CACTGATTC TTACCTATT	2000
MUSGLYTNs	1951	CTCTATGGCA AGTAATCAGG TATAATTCTT CAATGATTC TTATATATTC	2000
PCTCD	2001	.....	2050
BOVGSTA	2001	TTCTTCTCGG CCCCCCGGAA TCCATACACC ATCAGTTAA CC .....	2050
MUSGLYTNs	2001	TCCTCTCGG GAAATCTCA TTCTACAAAT CAUAAATTAT TTCAACAAAGC	2050
PCTCD	2051	.....	2100
BOVGSTA	2051	.....	2100
MUSGLYTNs	2051	AAAACCAAT GCGGAACT TCTTCAGT CTCTACAAAC ATTCACCACT	2100
PCTCD	2101	.....	2150
BOVGSTA	2101	.....	2150
MUSGLYTNs	2101	GGCCAGCTGC TCAGACACCC ATTACGGAAC ATCTTCCCTT CTGTCAAGCT	2150
PCTCD	2151	.....	2200
BOVGSTA	2151	.....	2200
MUSGLYTNs	2151	TGACACCTTC CATCCCTTA GCTTCATTAT CTTCCTCTC ATGGACATTC	2200
PCTCD	2201	.....	2250
BOVGSTA	2201	.....	2250
MUSGLYTNs	2201	TAAGGCAAC CAGACACCTT TACGACCCAG AGACCTTAA TAAAGATGCC	2250
PCTCD	2251	.....	2300
BOVGSTA	2251	.....	2300
MUSGLYTNs	2251	AACTCTACCA TCACCTCTA AAAGCTGATG CCTCCACCGA CGCTGCCAGT	2300
PCTCD	2301	.....	2350
BOVGSTA	2301	.....	2350
MUSGLYTNs	2301	CCAGGGCCCA CTAGCTATT CTTCCTCTC TCCCAATAA CCACCCACAC	2350
PCTCD	2351	.....	2400
BOVGSTA	2351	.....	2400
MUSGLYTNs	2351	AGTCCCAATA CCTACTTTCG TCCCACTATCT TCACAGTCGA CGAAUATCA	2400
PCTCD	2401	.....	2450
BOVGSTA	2401	.....	2450
MUSGLYTNs	2401	ACGCTACCA CCTACAGGA CATAAGCACTT CGGGTTGCTG ATTCACCA	2450
PCTCD	2451	.....	2500
BOVGSTA	2451	.....	2500
MUSGLYTNs	2451	CTTACAAACA CACACACCTT TCTTCAGGAT GACTAACAGC ACCAATGAA	2500

Fig. 4 (cont.) 4-5

PCTCD	2501 .....	.....	2550
BOVGSTA	2501 .....	.....	2550
MUSGLYTN3	2501 TCCAACTGTG CTTCATTTG TTTGCCAA ATTGTATTCG TCCGTAAAC	.....	2550
PCTCD	2551 .....	.....	2600
BOVGSTA	2551 .....	.....	2600
MUSGLYTN3	2551 TTTCTCTGTT GACCCCTGTC GACACGCTCT CACTGTATCA CGGACCCACA	.....	2600
PCTCD	2601 .....	.....	2650
BOVGSTA	2601 .....	.....	2650
MUSGLYTN3	2601 CTACCTCAGG CGACTCAGCA CGACCAACCT ATTATATTCAG AACACAACTG	.....	2650
PCTCD	2651 .....	.....	2700
BOVGSTA	2651 .....	.....	2700
MUSGLYTN3	2651 CTCATCAGCA CCTCTTACCT ACACCTCTC CTGAACTCTCC CACTTCTCA	.....	2700
PCTCD	2701 .....	.....	2750
BOVGSTA	2701 .....	.....	2750
MUSGLYTN3	2701 GCGCATCGTG TTTCAGCTGA GCGCCCTCTA TCCACCACT CACTGAGAC	.....	2750
PCTCD	2751 .....	.....	2800
BOVGSTA	2751 .....	.....	2800
MUSGLYTN3	2751 AAAACCCCTT GTCACATGAC CTCATTTCCC CTGCTCTAGT ACTATACAG	.....	2800
PCTCD	2801 .....	.....	2850
BOVGSTA	2801 .....	.....	2850
MUSGLYTN3	2801 TGTGACAGCC AGCCAGCCAG ATGTAATGCCA GACATAGCA ACCCACTTA	.....	2850
PCTCD	2851 .....	.....	2900
BOVGSTA	2851 .....	.....	2900
MUSGLYTN3	2851 TGGCAATGGG AGCCGAGTC ACTACAAAGG AGCTGCTCAA CCTTCCTGTC	.....	2900
PCTCD	2901 .....	.....	2950
BOVGSTA	2901 .....	.....	2950
MUSGLYTN3	2901 CGCGCTCTGA CACCTCTGAG GAGCCCTCTG ATAGCTGGTT CTGAACTAT	.....	2950
PCTCD	2951 .....	.....	3000
BOVGSTA	2951 .....	.....	3000
MUSGLYTN3	2951 CGCTCCCCAC CCTTTGGCA AGTGTAAAT GACCTTTCA CACCTCTCCC	.....	3000
PCTCD	3001 .....	.....	3050
BOVGSTA	3001 .....	.....	3050
MUSGLYTN3	3001 CTAAACGGT TAAAGAACAT AGATATTTCC ACTCTGACTG GTAAAGCTAG	.....	3050
PCTCD	3051 .....	.....	3100
BOVGSTA	3051 .....	.....	3100
MUSGLYTN3	3051 CAGAAATTACA GTTATCAAAAT AGCAAGGGAA ATAAATTCTGG CGTTGGTTC	.....	3100

# Fig 5

PBM Line-up (aa):

	Ex 4 ↓ Ex 5      Ex 5 ↓ Ex 6
PGT(Frame 1)	1 MNVKGRVVLS MLLVSTVMVV PHEYIINSPEC SLFWIYQSKN PEVG-GSAQR 50
BGT(Frame 1)	1' MNVKCKVILS MLLVSTVIIV PHEYIINSPEC SLFWIINPSRN PEVGGSSIQK 50
MGT(Frame 1)	1 MNVKCKVILL MLLVSTVVVV PHEYVNSPDC SFLWIYHTXI PEVGENRWQK 50
	Ex 6 ↓ Ex 7      Ex 7 ↓ Ex 8
PGT(Frame 1)	51 GWNFPSWFNN GTHSYNEEZD AIGNEXZQRK EDNRGELPLV DWFNPKERPE 100
BGT(Frame 1)	51 GWNLPWFNN G---YHEEDG DINEEKEQRN ED-ESXLKLS DWFNPKERPE 100
MGT(Frame 1)	51 DWWFPSWFNN GTHGYQEDRV EGRREK-CRN GDRIEEPQLW DWFNPKERPE 100
	Ex 8 ↓ Ex 9
PGT(Frame 1)	101 AVTITRKAP VVHECTYNRA VLDRNYYAKQK ITVGLTVFAV GRYIEIYLEZ 150
BGT(Frame 1)	101 VVTHTRKAP VVHECTYNRA VLDRNYYAKQK ITVGLTVFAV GRYIEIYLEZ 150
MGT(Frame 1)	101 VLTVTPWKAP IVHECTYDTA LLEKYYATQK LTGGLTVFAV GXYIEIYLEZ 150
PGT(Frame 1)	151 FLISANTYFH VGHKVIFYIM VDDISRMPLI ELCPLRSFKV FEIKSEKRWQ 200
BGT(Frame 1)	151 FLTSANKHFI VGHIPVIFYIM VDDVSRMPLI ELCPLRSFKV FKIXPEXRNQ 200
MGT(Frame 1)	151 FLEGACMYFH VGHRVIFYVM IDDTSRMPLV HLNPLHSLQV FEIRSEKRWQ 200
PGT(Frame 1)	201 DISMMRMKTI GEHLILAHIQH EVDFLFCMDV DQVFQNNFCV ETLGQSVQL 250
BGT(Frame 1)	201 DISMMRMKTI GEHLIVAHIQH EVDFLFCMDV DQVFQDKFCV ETLGQSVQL 250
MGT(Frame 1)	201 DISMMRMKTI GEHLILAHIQH EVDFLFCMDV DQVFQDNFCV ETLGQSVQL 250
PGT(Frame 1)	251 QAHWYKAHPD EFTYERRKES AAYIPFCQCD FYYHAAIFCC TPTQVLNITQ 300
BGT(Frame 1)	251 QAHWYKAOPH DFTYERRKES AAYIPFCQCD FYYHAAIFCC TPTQVLNITQ 300
MGT(Frame 1)	251 QAHWYKASPE KFTYERRELS AAYIPFCQCD FYYHAAIFCC TPTHILNLTR 300
PGT(Frame 1)	301 ECFKGILQDK ENDIEAEWHD ESHLNKYLIL NKPTKILSPE YCHDYHIGMS 350
BGT(Frame 1)	301 ECFKGILKDK KNDIEAQWHD ESHLNKYFLL NKPTKILSPE YCHDYHIGLP 350
MGT(Frame 1)	301 ECFKGILQDK KHDIEAQWHD ESHLNKYFLP NKPTKILSPE YCWDYQICLP 350
PGT(Frame 1)	351 VDIRIVKIAN QKREYNLVRN NI*..... 400
BGT(Frame 1)	351 ADIXLVIQMSW QTKEYNVVRN NV*..... 400
MGT(Frame 1)	351 SDIKSVKVAH QTKEYNLVRN NV*..... 400

Fig. 6.

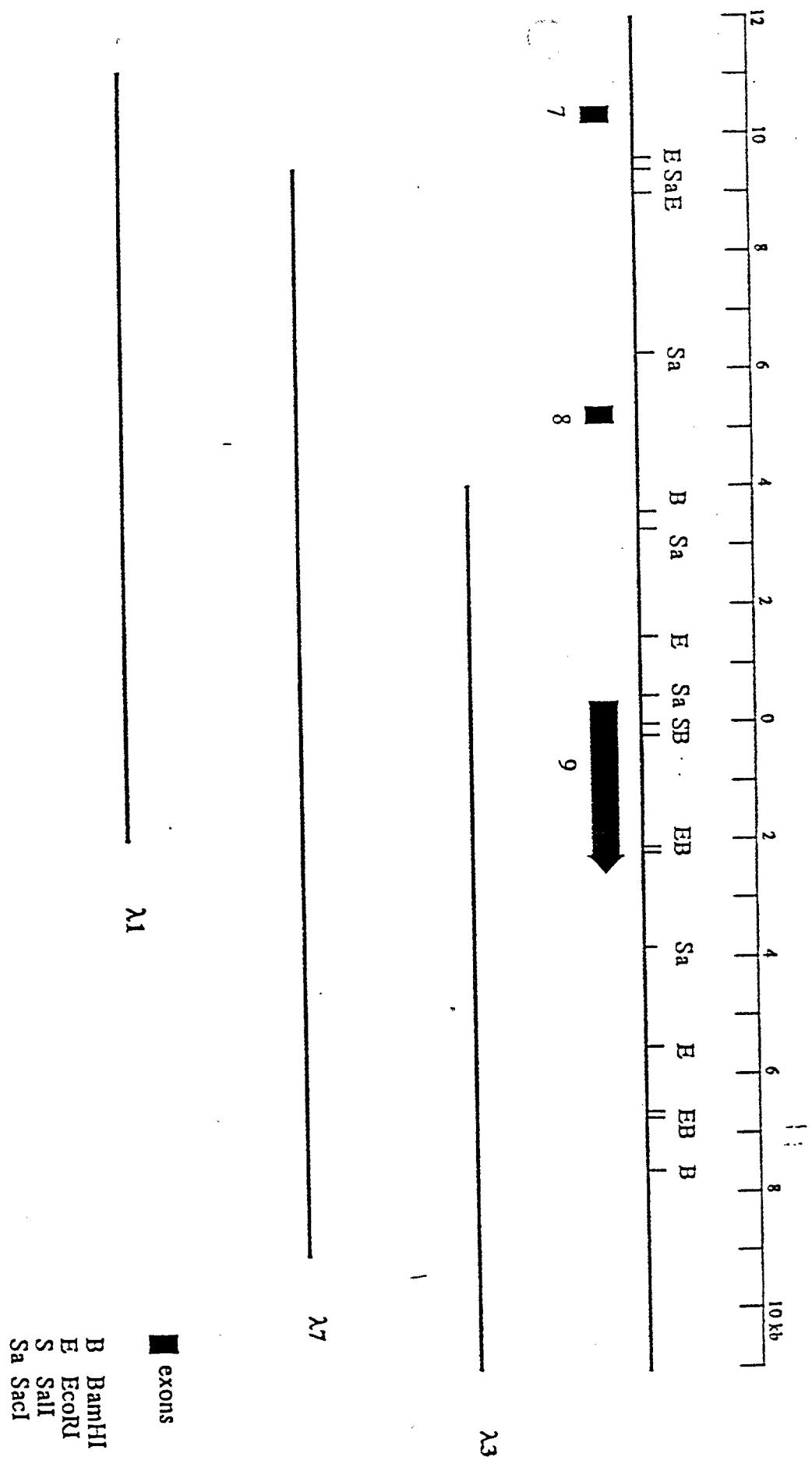
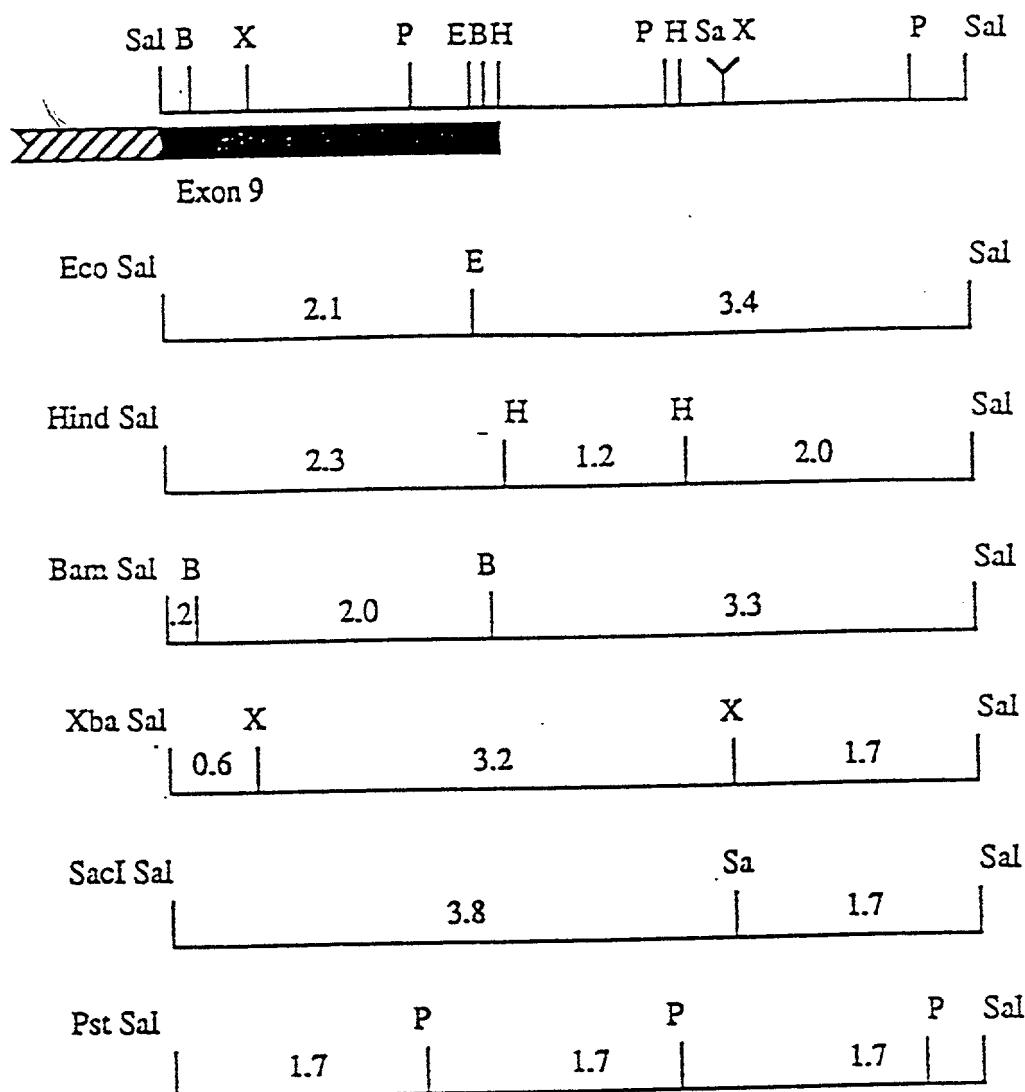


Fig. 7

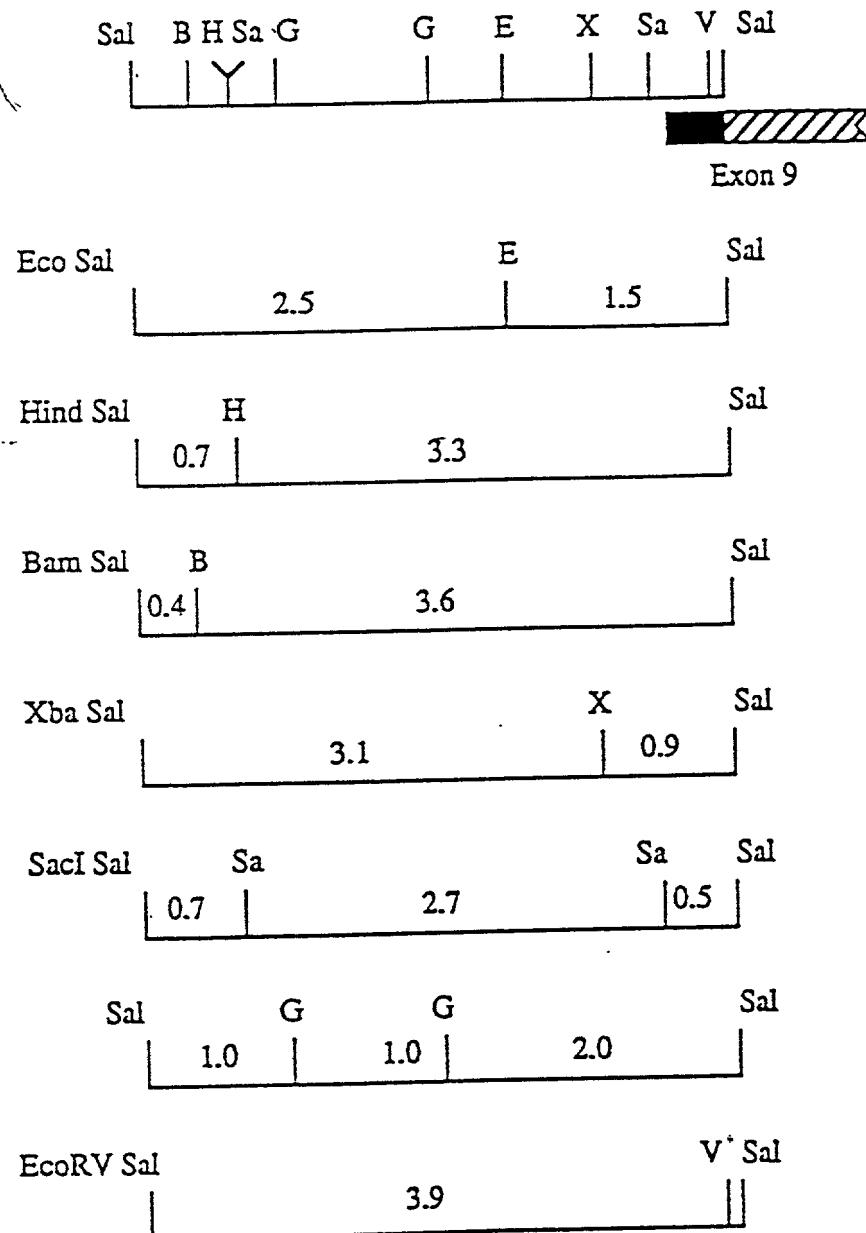


A	ApaI
B	BamHI
C	ClaI
E	EcoRI
G	BglII
H	HindIII
K	KpnI
N	NotI
P	PstI
S	SalI
Sa	SacI
V	EcoRV
X	XbaI
Xb	XbaII

No sites for: BglII, Nde, PvuI, Xba, Kpn, SacII, EcoRV, Sma, Cla, Apa, Not

pBS+KS:

.... SacI SacII Not Xba Spe Bam Sma Pst Eco RV Hind Cla Sal Xba Apa Kpn ...



A	ApaI
B	BamHI
C	ClaI
E	EcoRI
G	BglII
H	HindIII
K	KpnI
N	NotI
P	PstI
S	Sal
Sa	SacI
V	EcoRV
X	XbaI
Xh	XbaI

No sites for: Nde, PvuI, Xba, Kpn, SacII, Sma, Cla, Apa, Not

Unmapped sites for: Pst, PvuII

pUBS:

.... SacI SacII Not Xba Spe Bam Sma Pst Eco RV Hind Cla Sal Xba Apa Kpn ....

Fig. 9

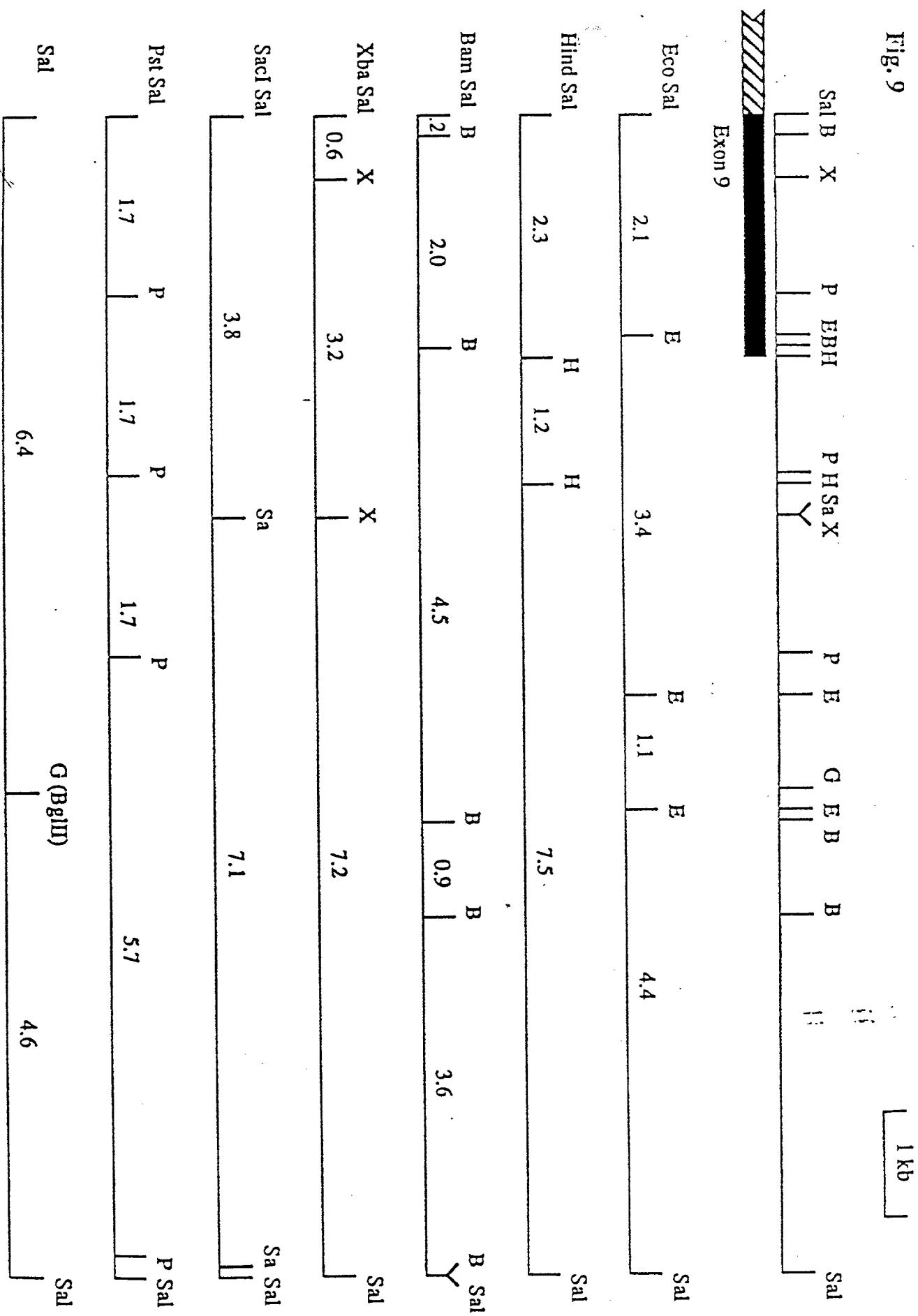


Fig. 9 (cont)

No sites for: Xba, Kpn, SacII, Sma, Cla, EcoRV, Apa, Not, PvuI, Nde  
pUBS:  
.... SacI SacII Not Xba Spe Bam Sma Pst Eco RV Hind Cla Sal Xba Apa Kpn ....



A ApaI  
B BamHI  
C ClaI  
E EcoRI  
G BglII  
H HindIII  
K KpnI  
N NotI  
P PstI  
S SalI  
Sa SacI  
V EcoRV  
X XbaI  
Xh XhoI

Fig. 10

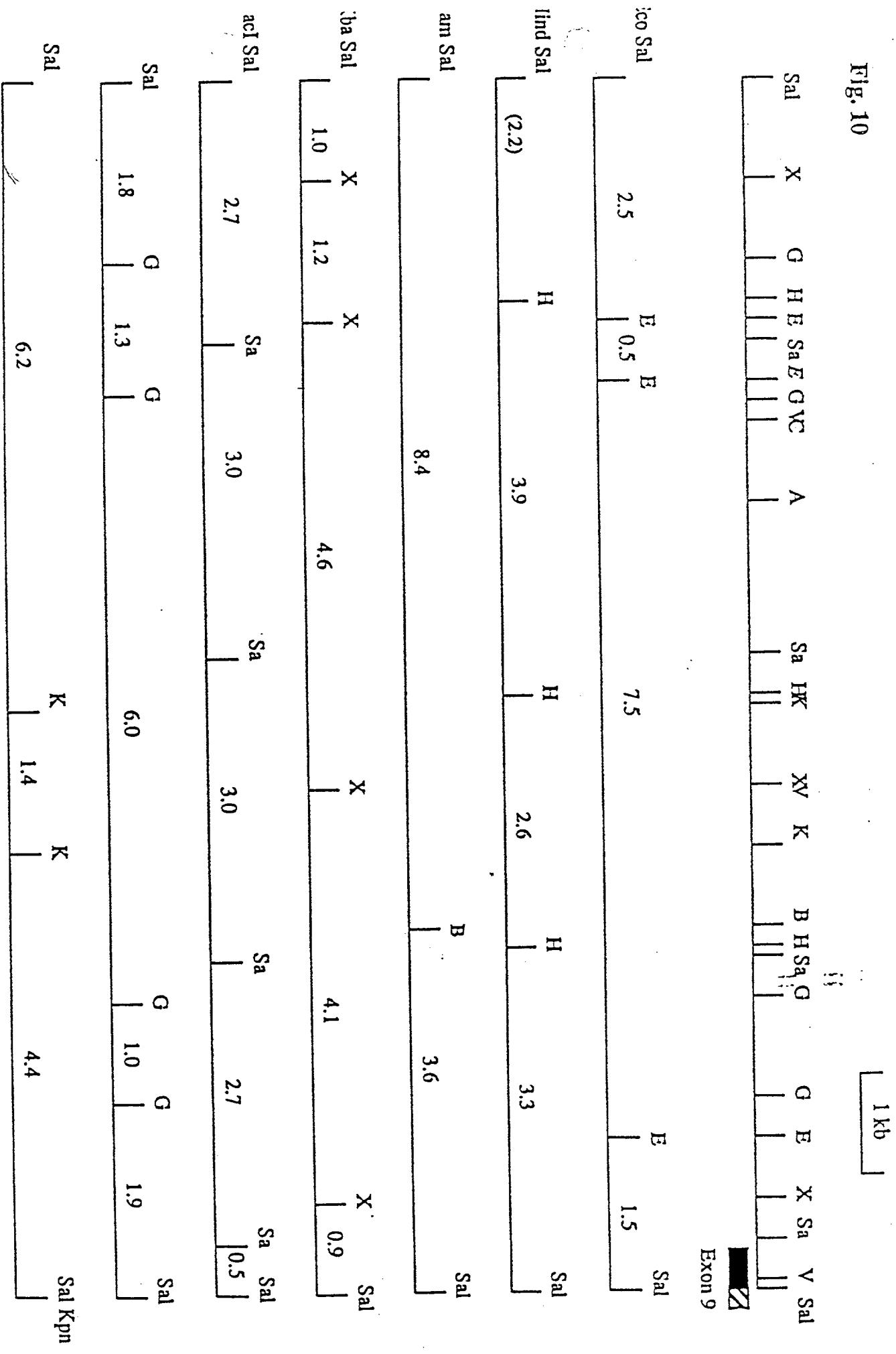
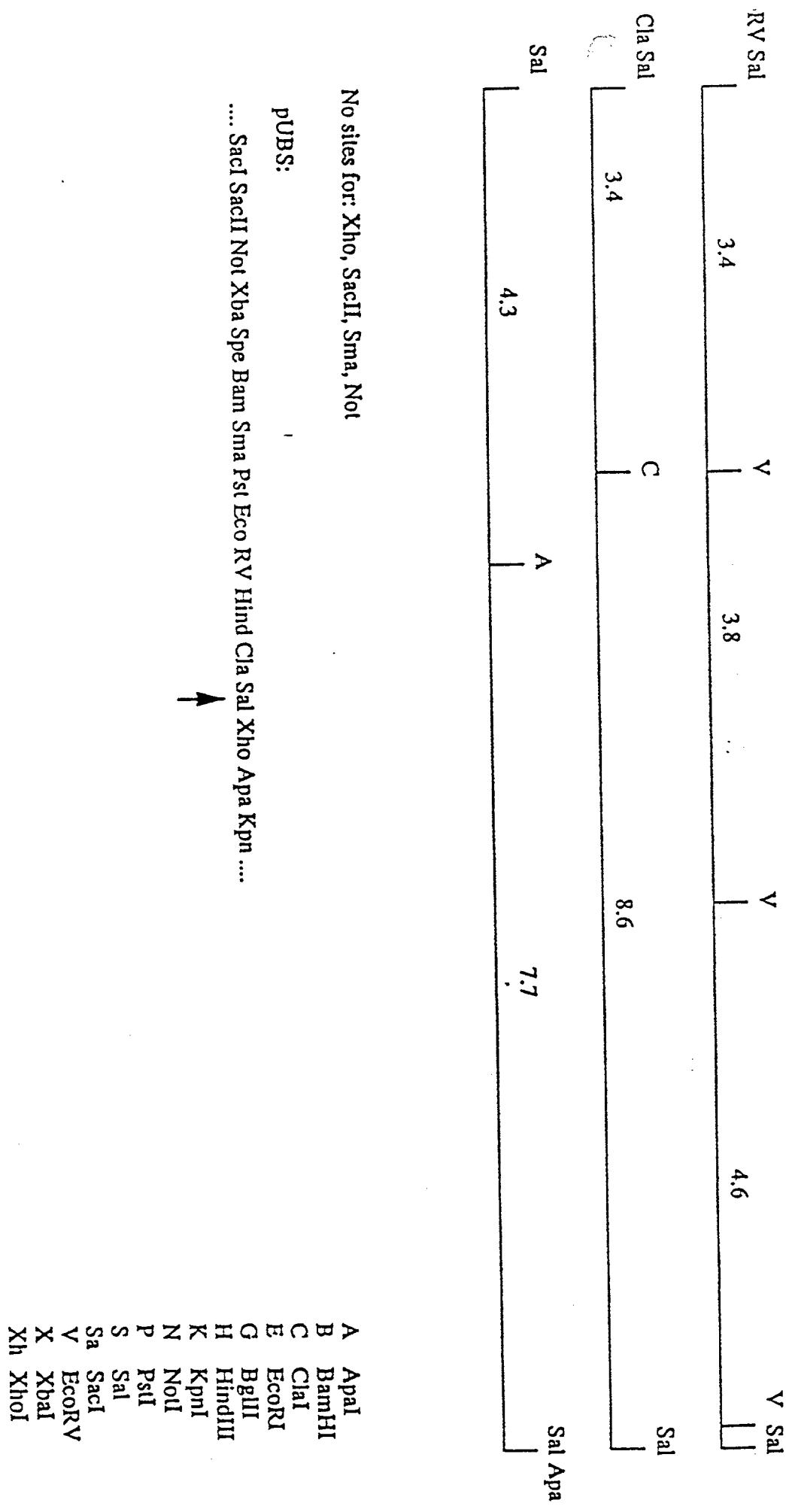
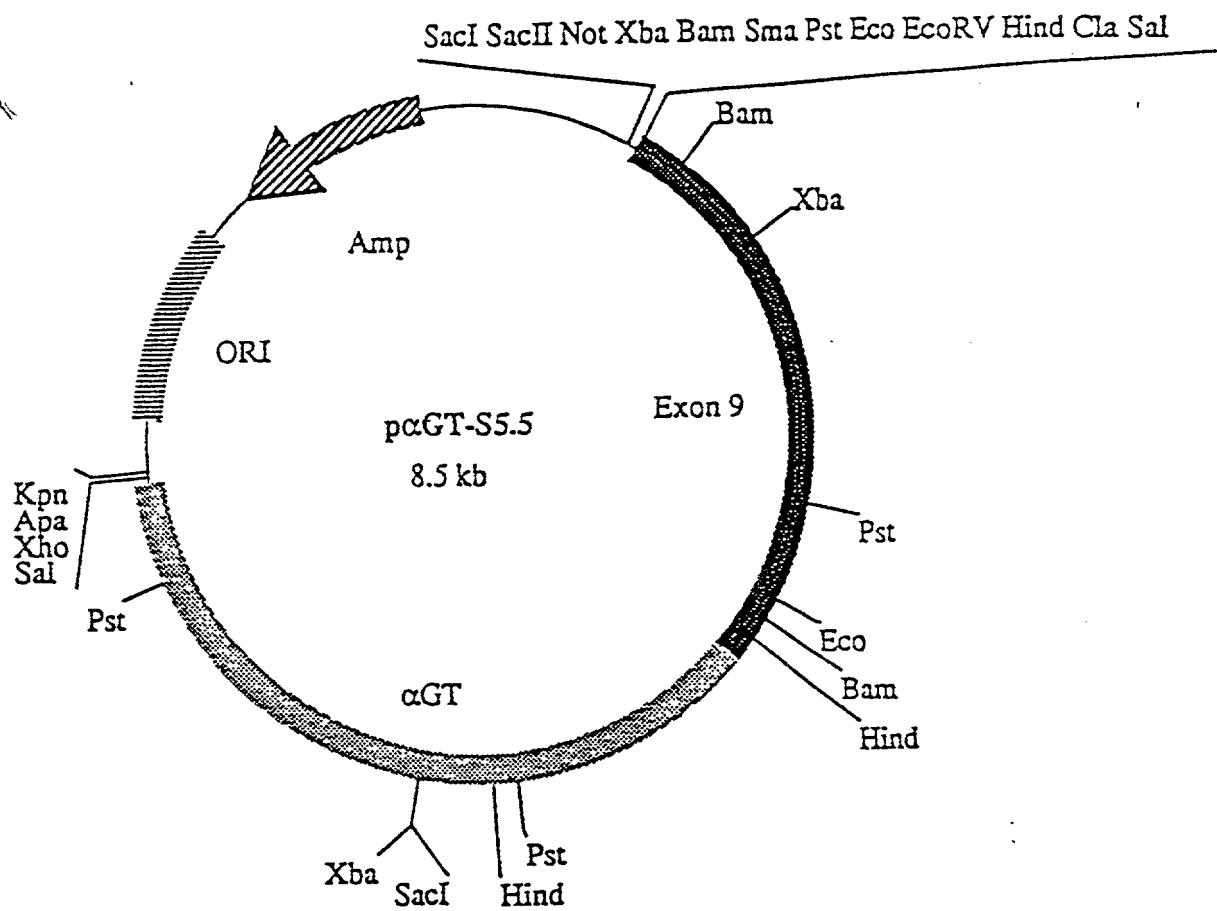
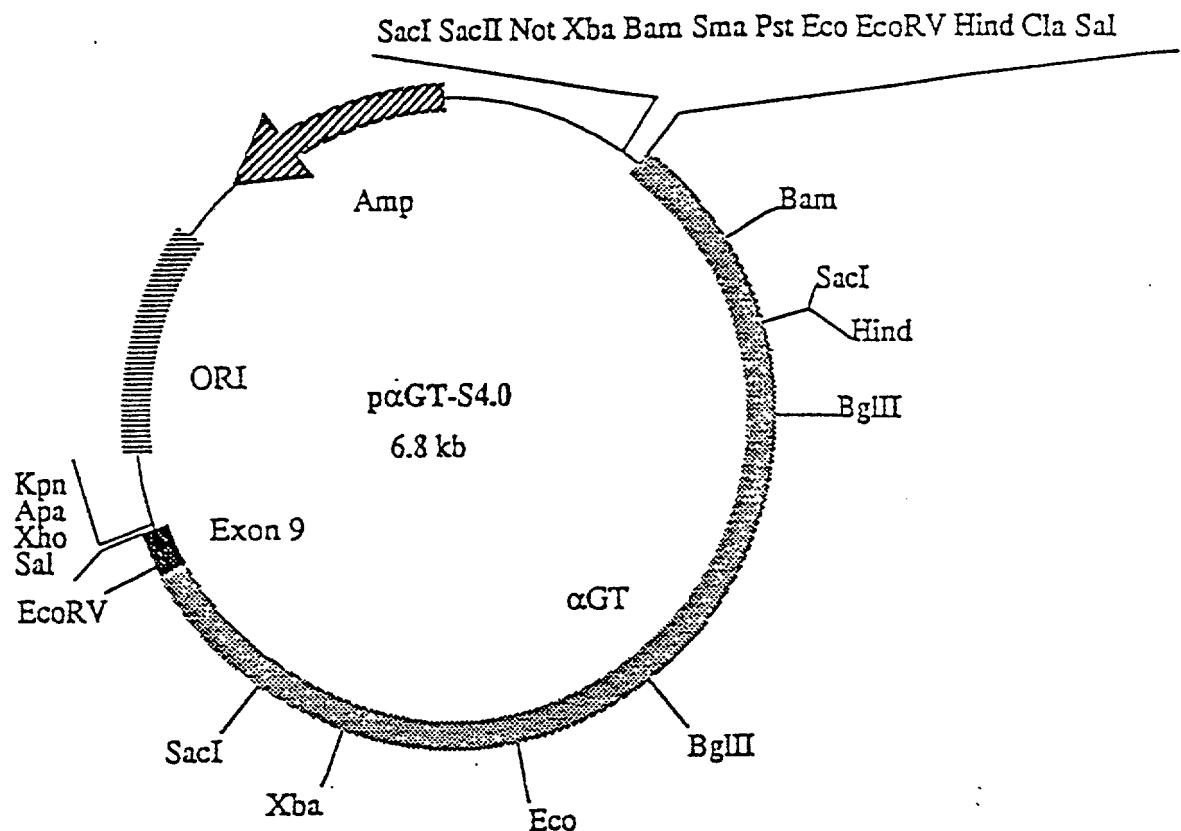


Fig. 10 (cont)





-   $\alpha$ 1,3 galactosyltransferase ( $\alpha$ GT)  
non-coding DNA
-  Exon 9 of  $\alpha$ GT
-  Neo resistance gene
-  Bacterial origin of replication
-  Amp resistance gene



-   $\alpha$ 1,3 galactotransferase ( $\alpha$ GT)  
non-coding DNA
-  Exon 9 of  $\alpha$ GT
-  Neo resistance gene
-  Bacterial origin of replication
-  Amp resistance gene

Fig. 13

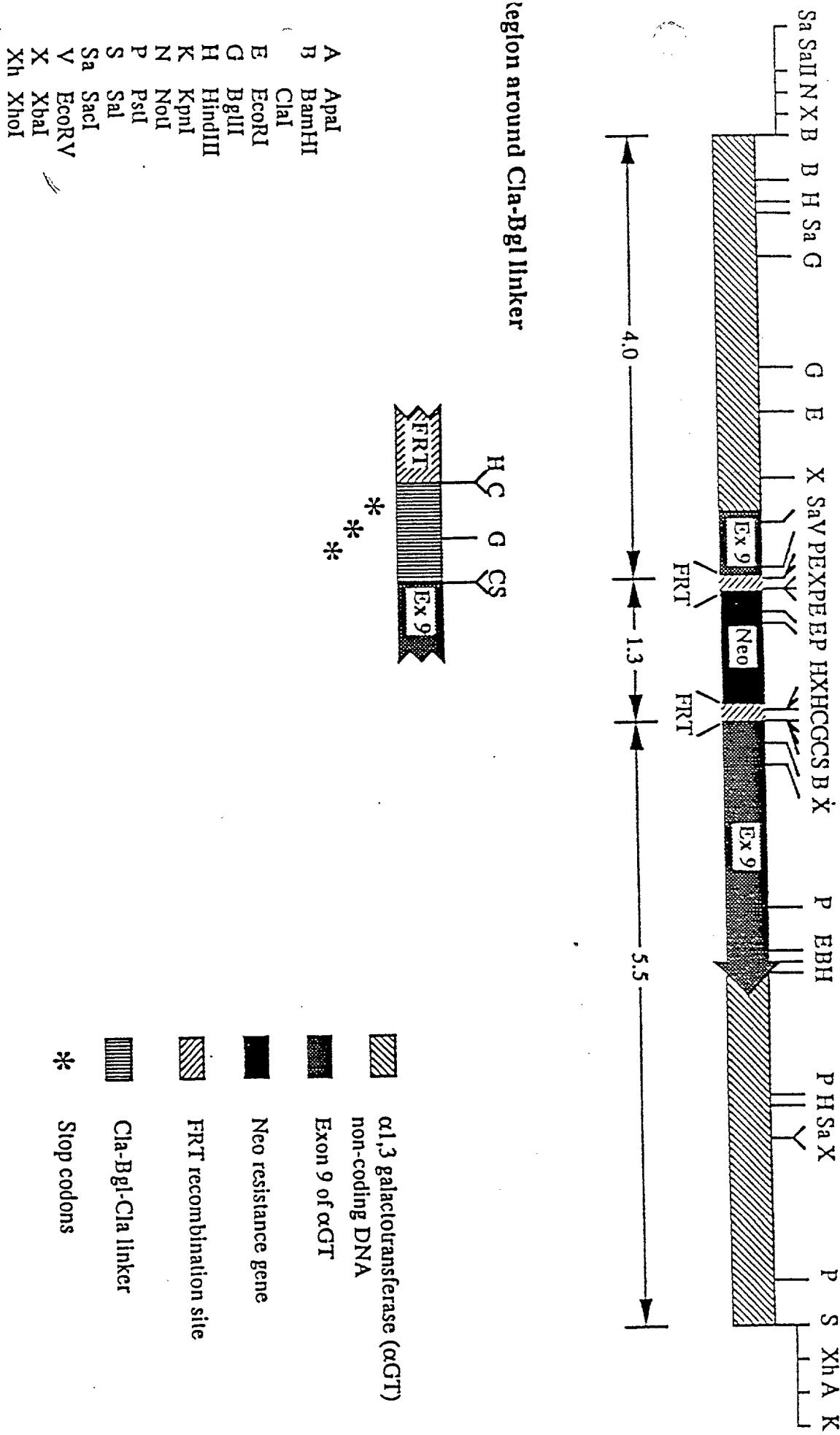


Fig. 14

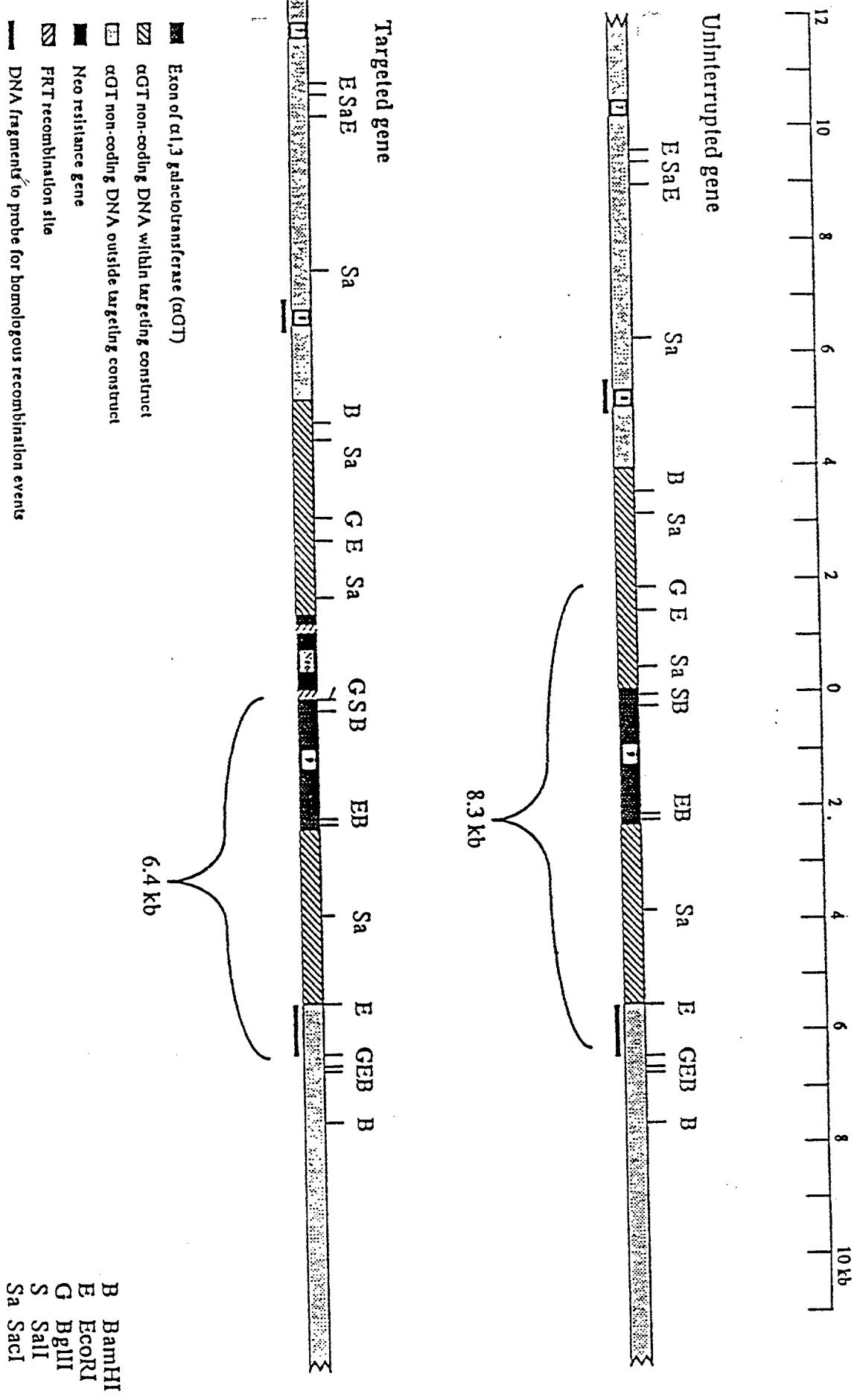
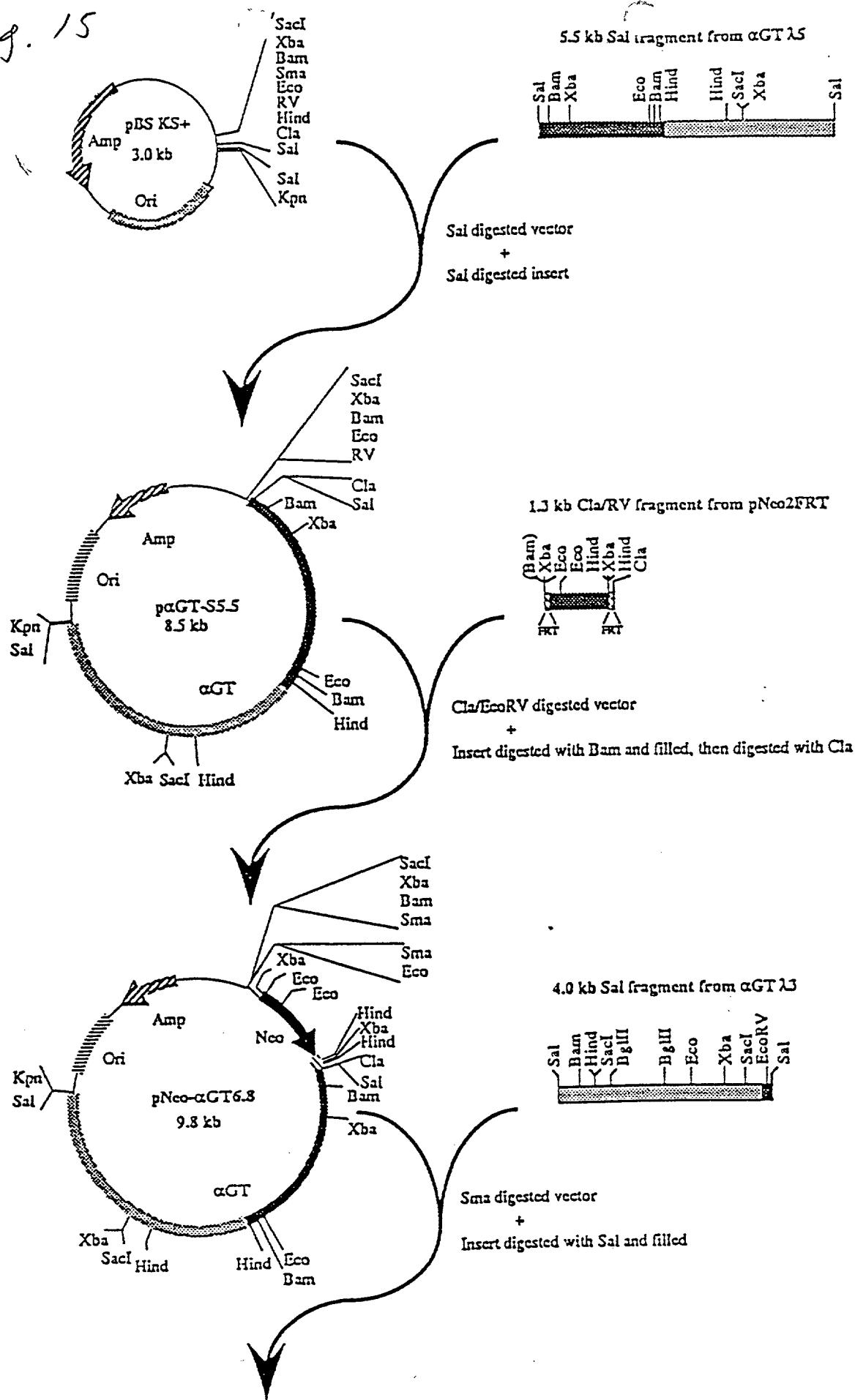
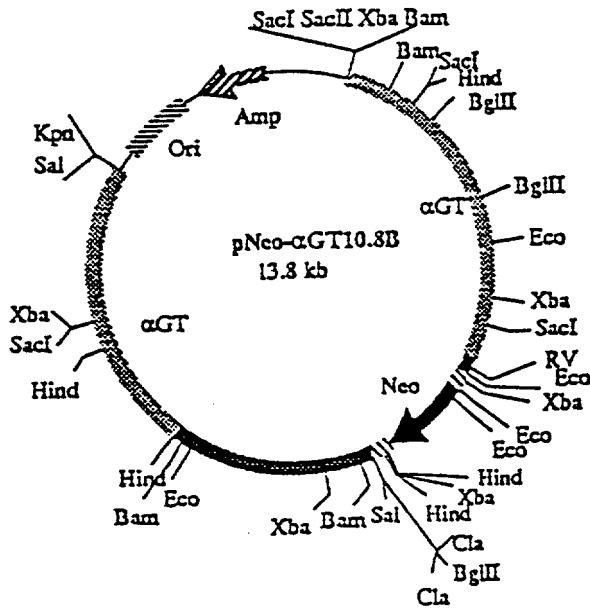
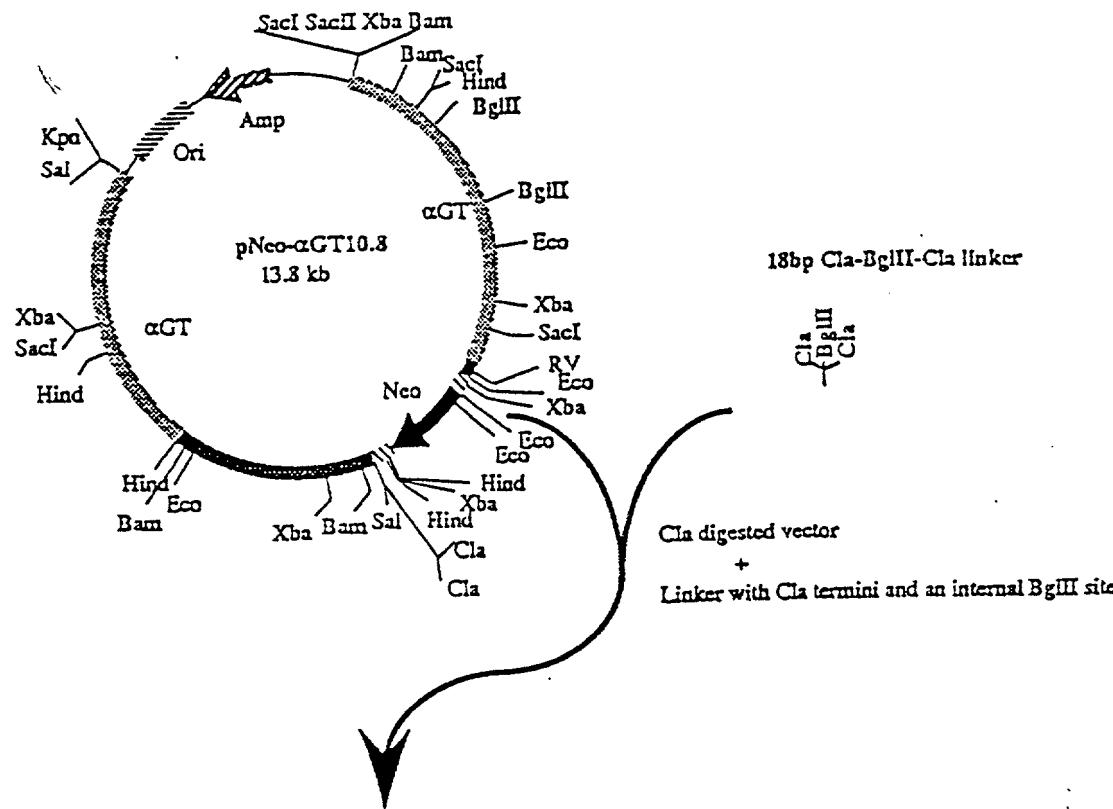


Fig. 15





- $\alpha$ 1,3 galactotransferase ( $\alpha$ GT) non-coding
- Exon 9 of  $\alpha$ GT
- Neo resistance gene
- FRT recombination site
- Bacterial origin of replication
- Amp resistance gene

Fig. 16

10 20 30 40 50 60  
GAGGGCTCGA CGAATTCCAT GATCCCCAG CTTGAAGTTC CTATTCGAA GTTCCTTATC  
70 80 90 100 110 120  
TCTAGAAAGT ATAGGAACCTT CAAGCTGGCC TGCAGGAATT CGATTCGAGC AGTGTGGTT  
130 140 150 160 170 180  
TGCAAGAGGA AGCAAAAGC CTCTCCACCC AGGCCTGGAA TGTTTCCACC CAATGTGAG  
190 200 210 220 230 240  
CACTGTGGTT TTGCAAGAGG AACCAAAAG CCTCTCCACC CAGGCCTGGAA ATGTTTCCAC  
250 260 270 280 290 300  
CCAATGTCCA CGAAACCCCG CCCAGCGTCT TGTCAATTGGC GAATTCGAC ACGGAGATGC  
310 320 330 340 350 360  
AGTCGGGGCG GCGCGGTCCC AGGTCCACTT GGCAATTTAA GGTGACGGGT GTGGCCTCGA  
370 380 390 400 410 420  
ACACCGAGCG ACCCTGAGC CAATATGGGA TCGGCCATTG AACAAAGATGG ATTGCACCC  
430 440 450 460 470 480  
GGTCTCTCCGG CGCGCTGGGT GGAGAGGCTA TTGGCTATG ACTGGGCACA ACAGACAAAC  
490 500 510 520 530 540  
GGCTGCTCTG ATGCCCGCGT GTTCCGGCTG TCAGGGCAGG GGCGCCCGGT TCCTTTTGTG  
550 560 570 580 590 600  
AAGACCGACC TGTCCGGTGC CCTGAATGAA CTCCAAGACG AGGCAGGGCG GCTATCGTGG  
610 620 630 640 650 660  
CTGGCCACGA CGGGCGTTC TTGGCAGCT GTGCTCGACG TTGTCACTGA AGCGGGAAAG  
670 680 690 700 710 720  
GAATGGCTGC TATTGGCGA AGTGGCGGGG CAGGATCTCC TGTCATCTCA CCTTGCTCTT  
730 740 750 760 770 780  
GCCGAGAAAG TATCCATCAT GGCTGATGCA ATGGGGGGC TGCATACGCT TGATCCGGCT  
790 800 810 820 830 840  
ACCTGCCAT TCGACCAACCA AGCGAAACAT CGCATCGAGC GAGCACGTAC TCGGATCGAA  
850 860 870 880 890 900  
GCCGGTCTTG TCGATCAGGA TGATCTGGAC GAAGAGCAGC AGGGGCTGGC GCCAGGGAA  
910 920 930 940 950 960  
CTGTTGCCA GGCTCAAGCC GCGGATGCC GACGGCGAGG ATCTCGTGT GACCCATGGC  
970 980 990 1000 1010 1020  
GATGGCTGCT TGCCGAATAT CATGGTGGAA AATGGCCGCT TTTCCTGGATT CATGGACTGT  
1030 1040 1050 1060 1070 1080  
GGCCGGCTGG GTGTGGCGGA CGCGTATCAG GACATACGCT TGGCTACCCG TGATATTGCT  
1090 1100 1110 1120 1130 1140  
GAAGAGCTTG CGGGCGAAATG GGCTGACCCG TTCCCTCGTGC TTTACGGTAT CGCCGGTCCC  
1150 1160 1170 1180 1190 1200  
GATTCGGAGC GCATCGCTT CTATCGCCTT CTTGACGGAGT TCTTCTGAGG CGATCGGAA  
1210 1220 1230 1240 1250 1260  
TAAAAAGACA GAATTAACCG CACGGGTGTT GGGCGTTTGT TCGGATCATC AAGCTGAA

Fig. 16 (cont )

1270 1280 1290 1300 1310 1320  
TTCCTATTCC GAAGTTCCCTA TTCTCTAGAA AGTATAGGAA CTTCAAGCTT ATCGATGAGT  
1330 1340 1350 1360 1370 1380  
AGATCTTGAT CGATACCGTC .....

Linker sequences : 0-28

FRT : 29-104

Polyoma virus enhancer repeats : 105-249

Herpes Simplex Virus Tyrosine Kinase promoter : 250-385

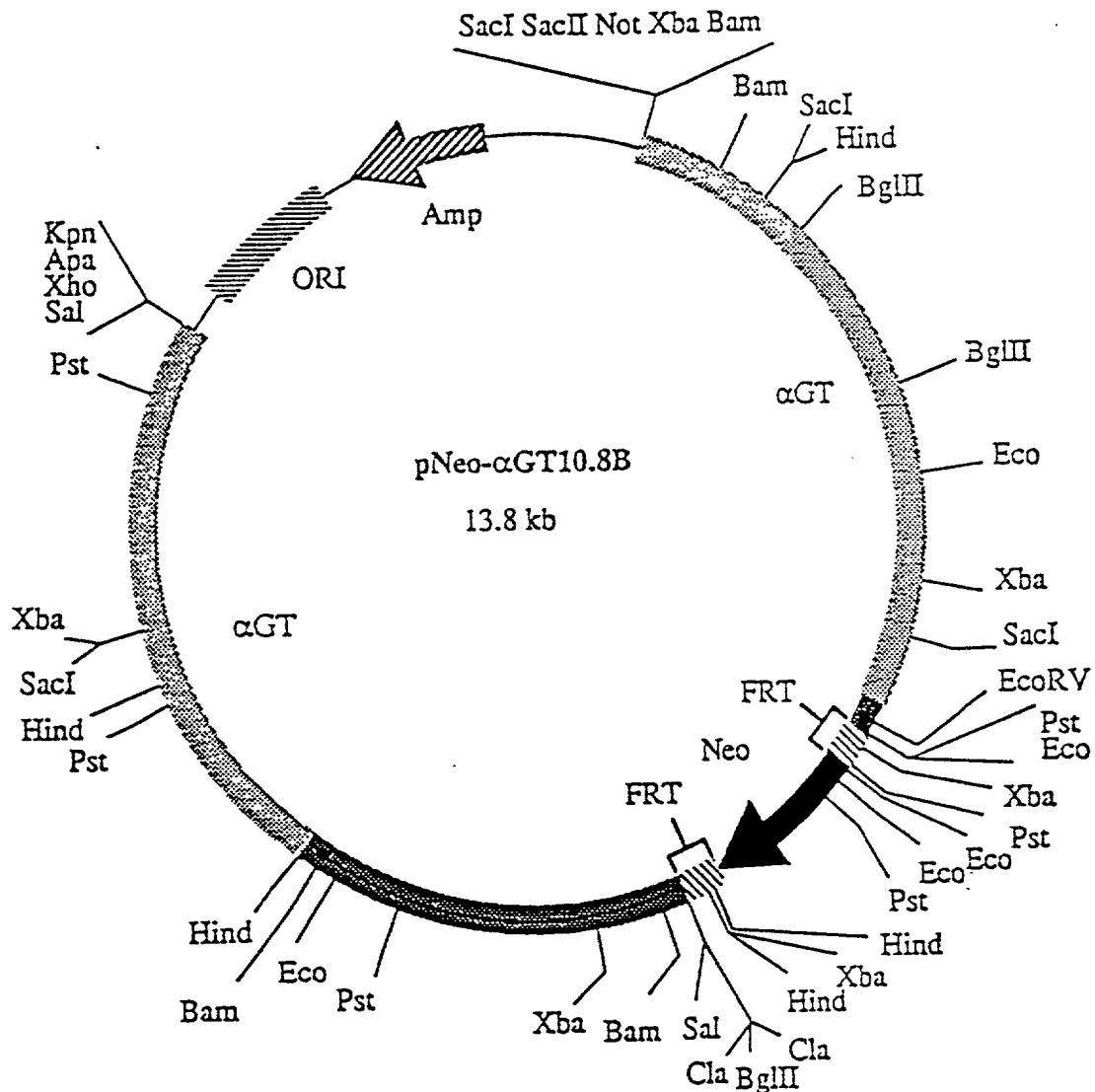
Neomycin phosphotransferase coding region : 385-1188

Herpes Simplex Virus Tyrosine Kinase PolyA signal : 1189-1249

FRT : 1250-1310

Linker sequences : 1311-1340

Fig. 17



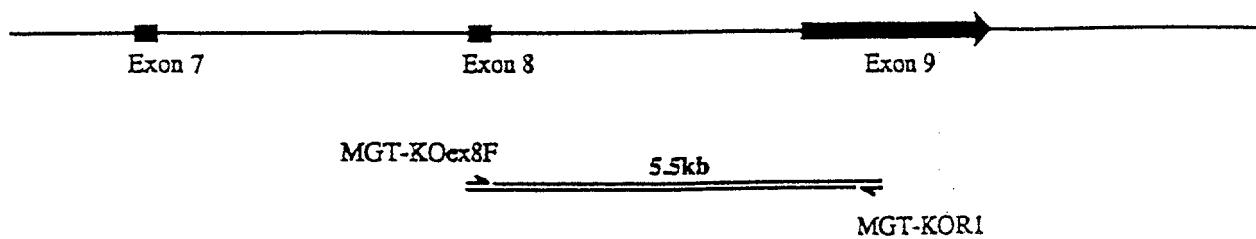
- $\alpha$ 1,3 galactotransferase ( $\alpha$ GT) non-coding DNA
- Exon 9 of  $\alpha$ GT
- Neo resistance gene
- FRT recombination site
- Bacterial origin of replication
- Amp resistance gene

Fig. 18



Fig. 17

Uninterrupted gene



Targeted gene

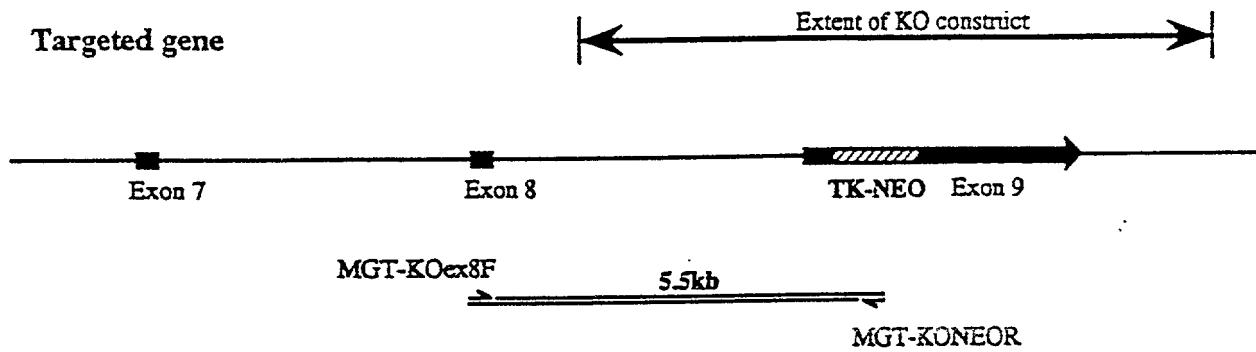
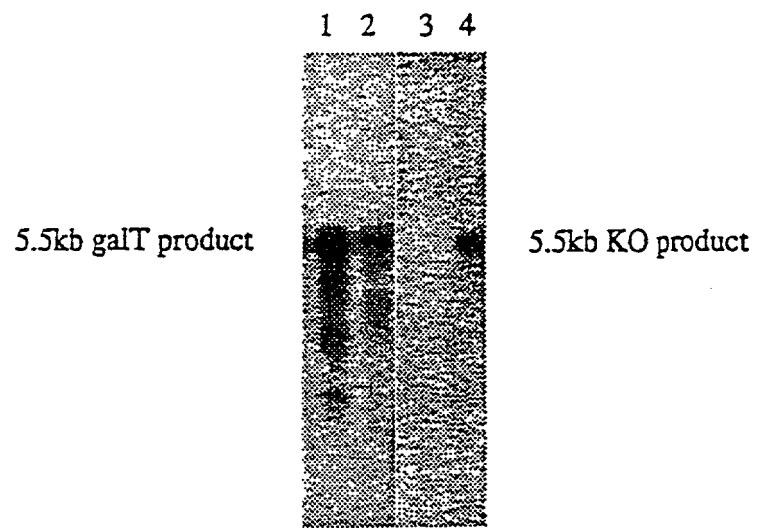


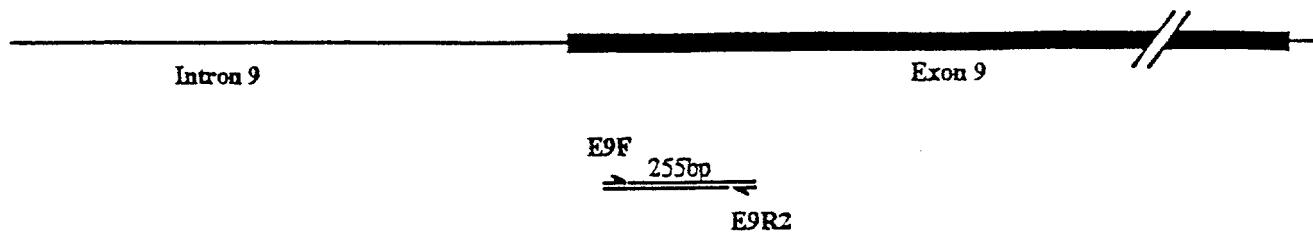
Fig.



1. CBAC template; wild type primers
2. 7C2 template; wild type primers
3. CBAC template; KO primers
4. 7C2 template; KO primers

Fig. 2/

Uninterrupted gene



Targeted gene

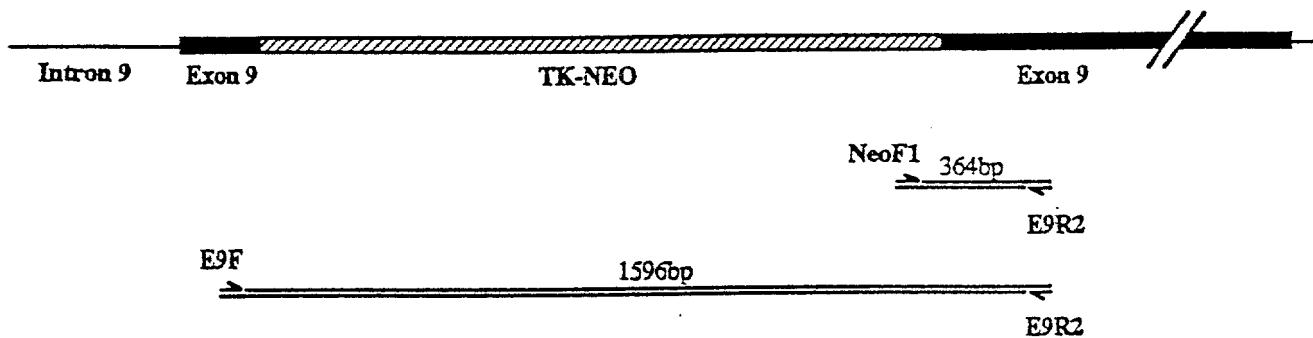


Fig. 22

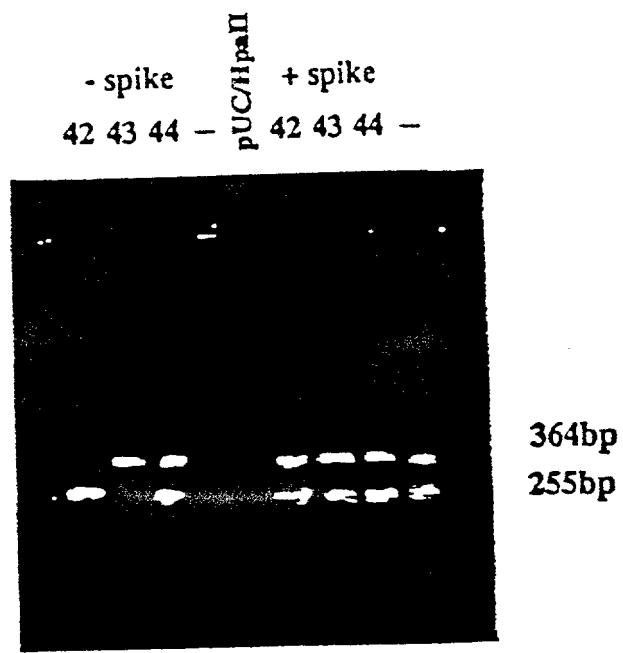
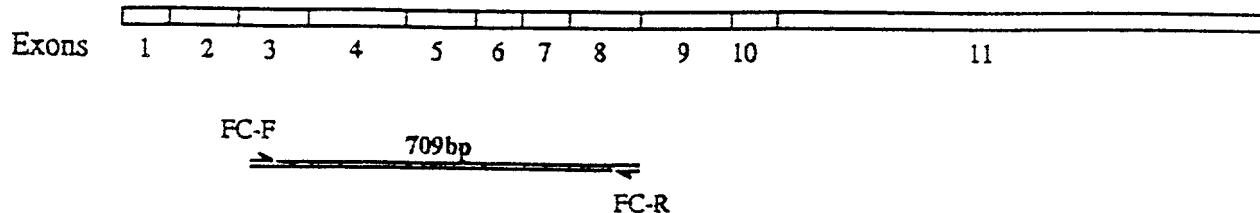
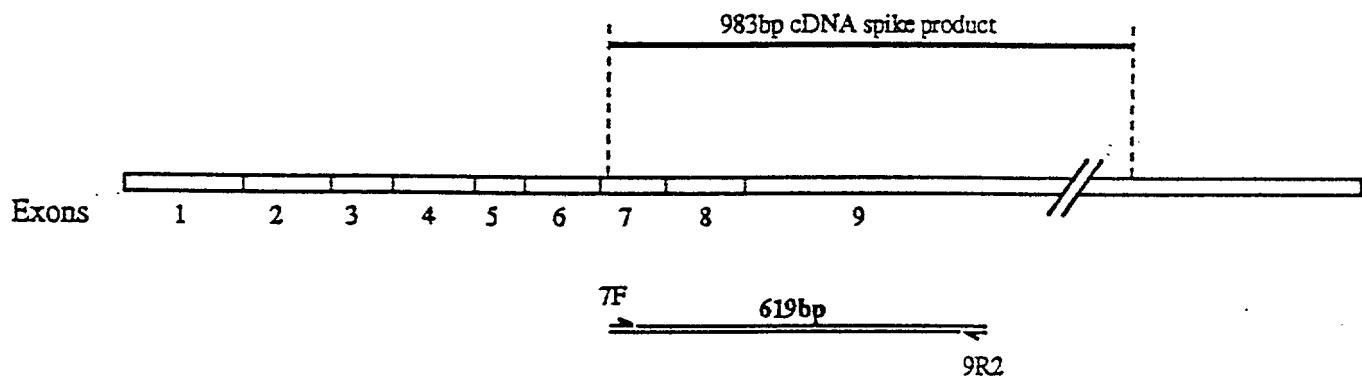


Fig. 23

Primer binding sites within mouse ferrochelatase cDNA



Primer binding sites within mouse  $\alpha$ -1,3-GaIT cDNA



## i) Ferrochelatase, FC-F/R

M, Marker SPP-I  
 C, MQW control  
 K, KIDNEY  
 H, HEART  
 L, LIVER

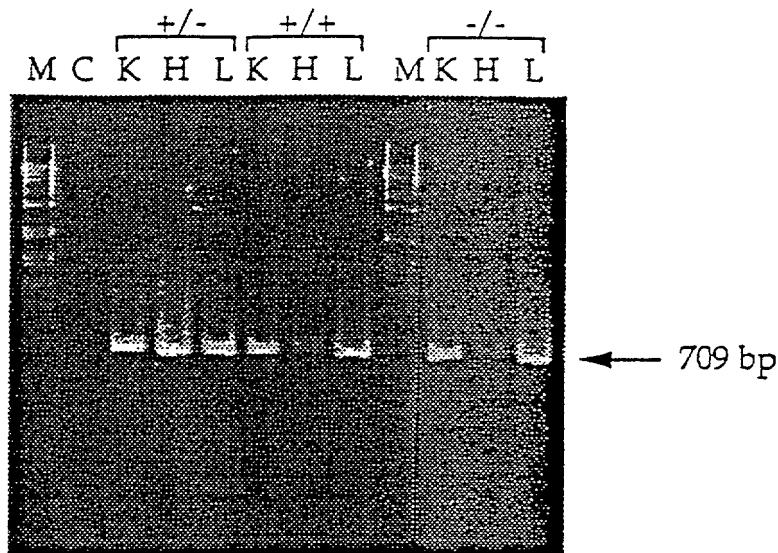
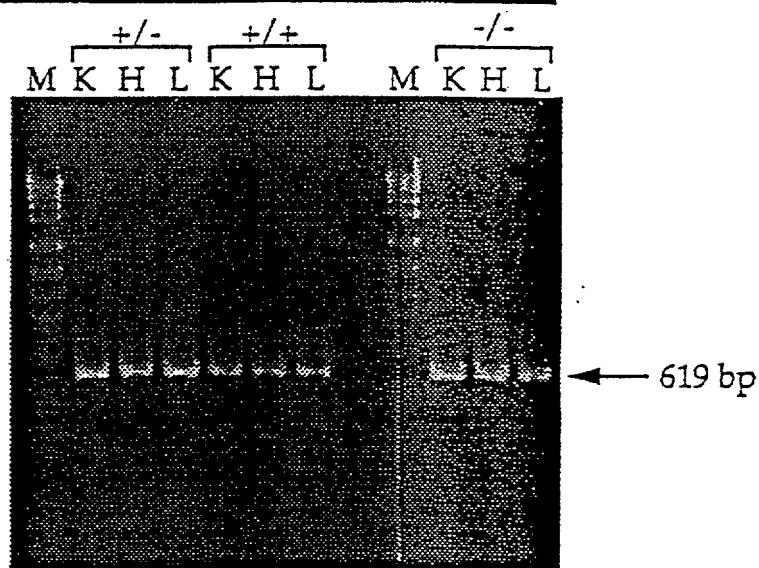
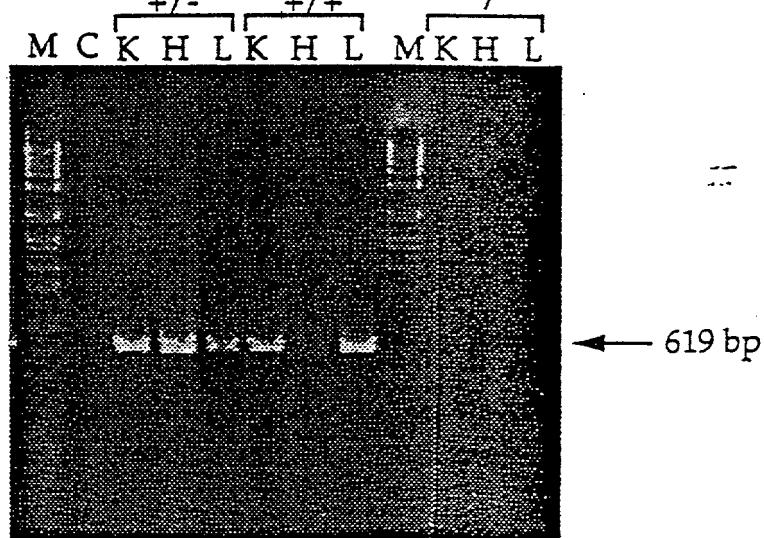
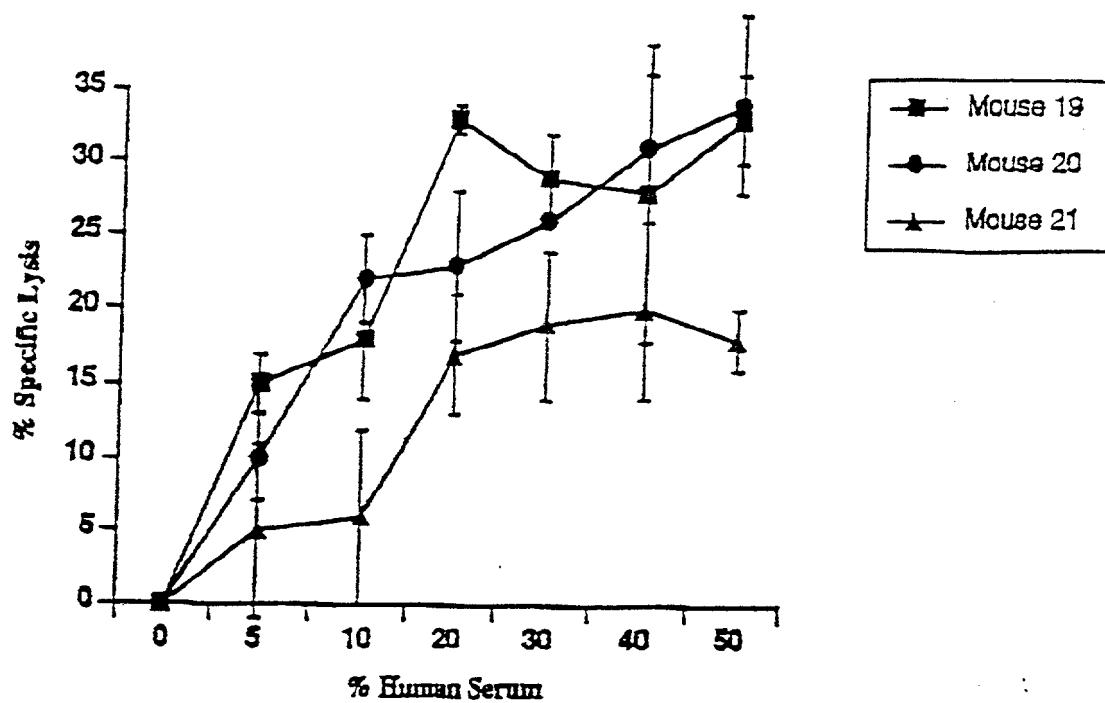
ii)  $\alpha$ -1,3-GT cDNA spike  
+ 7F/9R2 primersiii)  $\alpha$ -1,3-GT 7F/9R2 primers

Fig. 25



Mouse 19: Wild type; Mouse 20: Heterozygote Gal KO; Mouse 21:  
Homozygous Gal KO

Fig. 26

T-LIF SEQUENCE - Murine

CTGACACCTTCGCTTCCTCTGGGTCTCCGCTGCGACCTTCCCCACCC  
GGCCTCTTCCTGGTTGCACCTCCTCTCATTCCAAGGATTGCCCTTA  
CTGCTGCTGGTCTGCACTGGAAACACGGGGCAGGGAGCCCTCTTCCCATCAC  
CCCTGTAAATGCCACCTGTGCCATGCCACCCATGCCACGGCAACCTC

Met Asn Gln Ile Lys Asn Gln Leu Ala Gln Leu Asn Gly  
ATG AAC CAG ATC AAG AAT CAA CTG GCA CAG CTC AAT GGC

Ser Ala Asn Ala Leu Phe Ile Ser Tyr Tyr Thr Ala Gln Gly  
AGC GCC AAT GCT CTC TTC ATT TCC TAT TAC ACA GCT CAA GGX

Glu Pro Phe Pro Asn Asn Val Glu Lys Leu Cys Ala Pro Asn  
GAG CCG TTT CCC AAC AAC GTG GAA AAG CTA TGT GCG CCT AAC

Met Thr Asp Phe Pro Ser Phe His Gly Asn Gly Thr Glu Lys  
ATG ACA GAC TTC CCA TCT TTC CAT GGC AAC GGG ACA GAG AAG

Thr Lys Leu Val Glu Leu Tyr Arg Met Val Ala Tyr Leu Ser  
ACC AAG TTG GTG GAG CTG TAT CGG ATG GTC GCA TAC CTG AGC

Ala Ser Leu Thr Asn Ile Thr Arg Asp Gln Lys Val Leu Asn  
GCC TCC CTG ACC AAT ATC ACC CGG GAC CAG AAG GTC CTG AAC

Pro Thr Ala Val Ser Leu Gln Val Lys Leu Asn Ala Thr Ile  
CCC ACT GCC GTG AGC CTC CAG GTC AAG CTC AAT GCT ACT ATA

Asp Val Met Arg Gly Leu Leu Ser Asn Val Leu Cys Arg Leu  
GAC GTC ATG AGG GGC CTC CTC AGC AAT GTG CTT TGC CGT CTG

Cys Asn Lys Tyr Arg Val Gly His Val Asp Val Pro Pro Val  
TGC AAC AAG TAC CGT GTG GGC CAC GTG GAT GTG CCA CCT GTC

Pro Asp His Ser Asp Lys Glu Ala Phe Gln Arg Lys Lys Leu  
CCC GAC CAC TCT GAC AAA GAA GCC TTC CAA AGG AAA AAG TTG

Gly Cys Gln Leu Leu Gly Thr Tyr Lys Gln Val Ile Ser Val  
GGT TGC CAG CTT CTG GGG ACA TAC AAG CAA GTC ATA AGT GTG

Val Val Gln Ala Phe \*\*\*  
GTC GTC CAG GCC TTC TAG AGAGGAGGTCTGAATGTACCATGGACTG...

Fig. 27

HUMAN T-LIF SEQUENCE

GACCTTTGC	CTTTCTCTC	TCCTGGTGCA	CCATTTCCCTC	TCCCTCCCTG	50
AGCCGGAGTT	GTGCCCTGC	TGTTGGTTCT	GCACTGGAAA	CATGGGGCGG	100
GGAGCCCCCT	CCCCATCACC	CCTGTCAACG	CCACCTGTGC	CATACGCCAC	150
CCATGTCACA	ACAAACCTC	ATG AAC CAG ATC			182
Met Asn Gln Ile					
AGG AGC CAA CTG GCA CAG CTC AAT GGC AGT GCC AAT GCC CTC					227
Arg Ser Gln Leu Ala Gln Leu Asn Gly Ser Ala Asn Ala Leu					
5	10		15		
TTT ATT CTC TAT TAC ACA GCC CAG GGG GAG CCG TTC CCC AAC					272
Phe Ile Leu Tyr Tyr Thr Ala Gln Gly Glu Pro Phe Pro Asn					
20	25		30		
AAC CTG GAC AAG CTA TGT GGC CCC AAC GTG ACG GAC TTC CCG					317
Asn Leu Asp Lys Leu Cys Gly Pro Asn Val Thr Asp Phe Pro					
35	40		45		
CCC TTC CAC GCC AAC GGC ACG GAG AAG GCC AAG CTG GTG GAG					362
Pro Phe His Ala Asn Gly Thr Glu Lys Ala Lys Leu Val Glu					
50	55		60		
CTG TAC CGC ATA GTC GTG TAC CTT GGC ACC TCC CTG GGC AAC					407
Leu Tyr Arg Ile Val Val Tyr Leu Gly Thr Ser Leu Gly Asn					
65	70				
ATC ACC CGG GAC CAG AAG ATC CTC AAC CCC AGT GCC CTC AGC					452
Ile Thr Arg Asp Gln Lys Ile Leu Asn Pro Ser Ala Leu Ser					
75	80		85		
CTC CAC AGC AAG CTC AAC GCC ACC GCC GAC ATC CTG CGA GGC					497
Leu His Ser Lys Leu Asn Ala Thr Ala Asp Ile Leu Arg Gly					
90	95		100		
CTC CTT AGC AAC GTG CTG TGC CGC CTG TGC AGC AAG TAC CAC					542
Leu Leu Ser Asn Val Leu Cys Arg Leu Cys Ser Lys Tyr His					
105	110		115		
GTG GGC CAT GTG GAC GTG ACC TAC GGC CCT GAC ACC TCG GGT					587
Val Gly His Val Asp Val Thr Tyr Gly Pro Asp Thr Ser Gly					
120	125		130		
AAG GAT GTC TTC CAG AAG AAG CTG GGC TGT CAA CTC CTG					632
Lys Asp Val Phe Gln Lys Lys Lys Leu Gly Cys Gln Leu Leu					

Fig. 27 (cont.)

27-2

135	140	
GGG AAG TAT AAG CAG ATC ATC GCC GTG TTG GCC CAG GCC TTC	677	
Gly Lys Tyr Lys Gln Ile Ile Ala Val Leu Ala Gln Ala Phe		
145	150	155
TAG CAGGAGGTCT		722
***		

Fig. 28

(29)

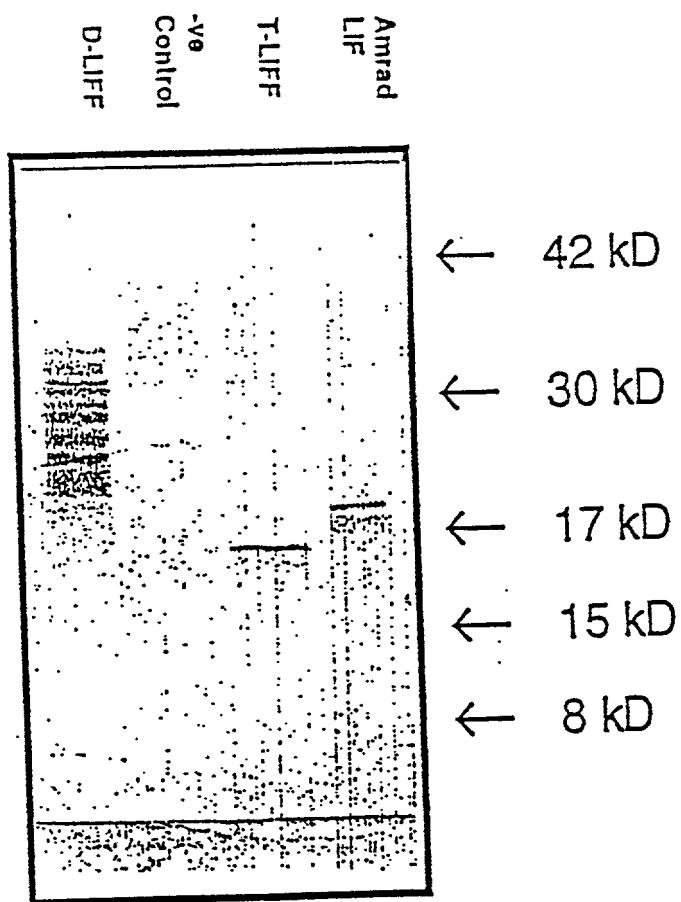
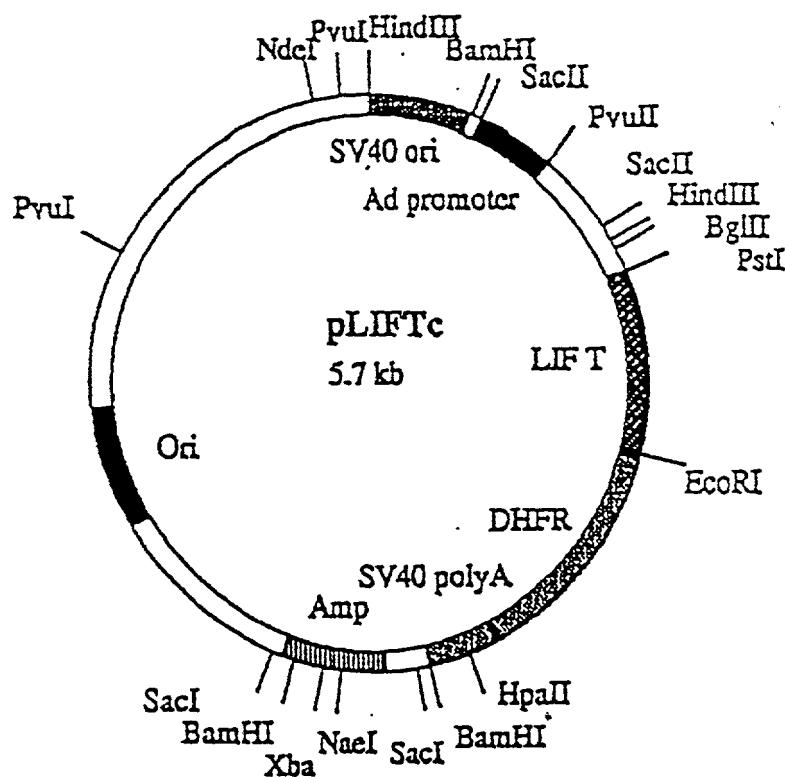


Fig. 29

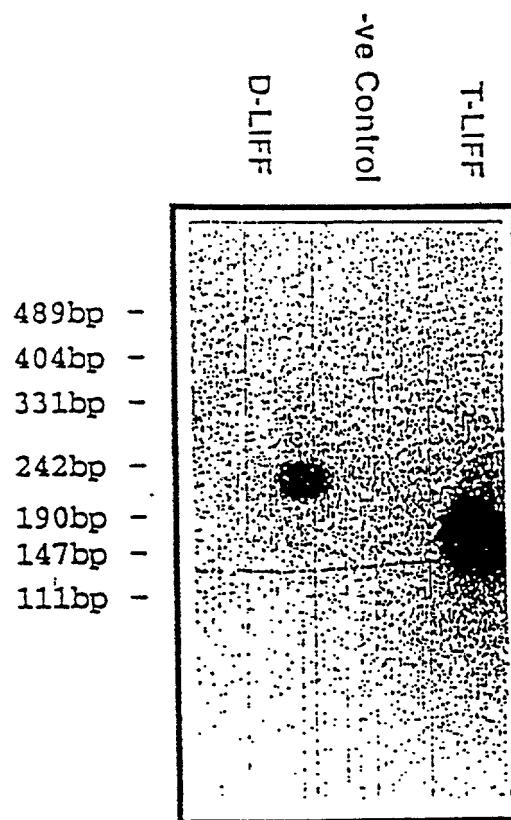
(30)



- SV40 origin of replication
- Adenovirus promoter
- LIFT coding region
- Dihydrofolate reductase 3' end
- Ampicillin resistance gene
- Bacterial origin of replication

Fig. 30

(31)



**COMBINED DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled MATERIALS AND METHODS FOR MANAGEMENT OF HYPERACUTE REJECTION IN HUMAN XENOTRANSPLANTATION, the specification of which

is attached hereto.  
 was filed on January 26, 1995 as Application Serial No. 08/378,617 and was amended on \_\_\_\_\_.  
 was described and claimed in PCT International Application No. \_\_\_\_\_ filed on \_\_\_\_\_ and as amended under PCT Article 19 on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Mark S. Ellinger, Reg. No. 34,812, William E. Booth, Reg. No. 28,933; Karl Bozicevic, Reg. No. 28,807; Barry E. Bretschneider, Reg. No. 28,055; Paul T. Clark, Reg. No. 30,162; Peter J. Devlin, Reg. No. 31,753; Willis M. Ertman, Reg. No. 18,658; David L. Feigenbaum, Reg. No. 30,378; Janis K. Fraser, Reg. No. 34,819; John W. Freeman, Reg. No. 29,066; Timothy A. French, Reg. No. 30,175; Alan H. Gordon, Reg. No. 26,168; Scott C. Harris, Reg. No. 32,030; Mark J. Hebert, Reg. No. 31,766; Gilbert H. Hennessey, Reg. No. 25,759; Charles Hieken, Reg. No. 18,411; Robert E. Hillman, Reg. No. 22,837; G. Roger Lee, Reg. No. 28,963; Steven E. Lipman, Reg. No. 30,011; Gregory A. Madera, Reg. No. 28,878; Ralph A. Mittelberger, Reg. No. 33,195; Ronald E. Myrick, Reg. No. 26,315; Robert C. Nabinger, Reg. No. 33,431; Frank P. Porcelli, Reg. No. 27,374; Eric L. Prahl, Reg. No. 32,590; Alan D. Rosenthal, Reg. No. 27,833; Richard M. Sharkansky, Reg. No. 25,800; John M. Skenyon, Reg. No. 27,468; Michael O. Sutton, Reg. No. 26,675; Rene D. Tegtmeyer, Reg. No. 33,567; Hans R. Troesch, Reg. No. 36,950; John N. Williams, Reg. No. 18,948; Gary A. Walpert, Reg. No. 26,098; Dorothy P. Whelan, Reg. No. 33,814; Charles C. Winchester, Reg. No. 21,040; Thomas S. Borecki, Reg. No. 32,964 and Charles Mattenson, Reg. No. 30,660.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

## COMBINED DECLARATION AND POWER OF ATTORNEY CONTINUED

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Post Office Address: Balwyn, Victoria, Australia

Full Name of Inventor: Martin J. Pearse

Inventor's Signature: M. Pearse

Date: 5/4/95

Residence Address: Mordialloc Victoria 3195 AUSTRALIA

Citizen of: Australia

Post Office Address: 37 Chute Street, Mordialloc Victoria, 3195 AUSTRALIA

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled MATERIALS AND METHODS FOR MANAGEMENT OF HYPERACUTE REJECTION IN HUMAN XENOTRANSPLANTATION, the specification of which

is attached hereto.  
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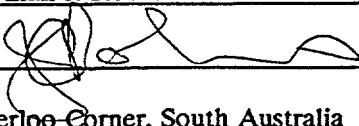
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## COMBINED DECLARATION AND POWER OF ATTORNEY CONTINUED

Full Name of Inventor: Allan J. Robins

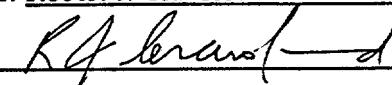
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